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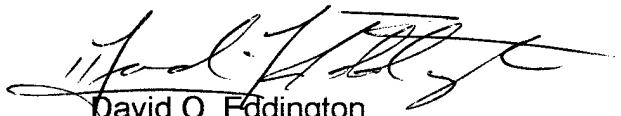

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ABSTRACT

Title: Localization and Expression of the Proto-oncoprotein *Brx* in the Mouse Brain and Pituitary

Author: David O. Eddington

Thesis directed by: Dr. T. John Wu,
Department of Obstetrics and Gynecology

A new member of the Dbl family of oncoproteins was discovered in breast cancer tissue extracts. This novel protein, designated Brx, contains an estrogen-receptor binding motif and is highly expressed in hormone-responsive breast tissue. Due to its ability to augment ligand-dependent activation of estrogen receptors, we analyzed the expression of Brx in the adult mouse brain and pituitary. Results indicated that Brx is expressed in specific regions of the brain and pituitary. Furthermore, the results indicate that differences exist in both brain and pituitary tissue of male and female mice with greater expression in the female. However, estrogen was not able to regulate Brx expression in ovariectomized mice. The anatomical studies support a role for Brx in its association with the estrogen receptor and that Brx may be involved in neuronal and pituitary function in a sexually dimorphic manner.

LOCALIZATION AND EXPRESSION OF THE PROTO-ONCOPROTEIN *BRX* IN THE MOUSE BRAIN AND PITUITARY

By

David O. Eddington, Captain, USAF

Thesis submitted to the Faculty of the
Molecular and Cellular Biology Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Master of Science 2003

DEDICATION

This work is the culmination of a great deal of effort. It may not have come to fruition without the support, both intentional and unintentional, that my family has given me. I dedicate this labor to them. First, and foremost, I dedicate this to my wife, Shelly, because she always believed in me, even during times I wasn't so sure I believed in myself. Her continuous love and encouragement kept me afloat through the good and through the not so good. Thank you my dear, I couldn't have done it without you! Secondly, I dedicate this project to Chad, Julia, Caroline, Marissa, and Annaliese. Thank you for giving me perspective when I most needed it. May you discover early in life that learning is a continuous process and the constant pursuit of knowledge (when tempered) can bring much fulfillment to your lives. I also wish to dedicate this work to my parents. They molded me at an impressionable age, provided me with excellent examples to follow, taught me about priorities, and helped me to "Aim High". I was enormously fortunate to have been nurtured by you both. And, finally, to my brother Kenneth who is an enduring friend and whose shared love of biological sciences has allowed us to mutually encourage each other in the field. Thank you for being my brother, my friend, and my colleague.

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There were many who influenced my work and provided valuable assistance who I wish to acknowledge here. First, my thesis advisor, Dr. T. John Wu, who gave me the privilege of working in his lab, for his constant guidance and assistance, for shaping this work, and for shaping me as a scientist. He was always there to support and mentor. Next, I acknowledge the indispensable assistance given by Dr. William Gause. I couldn't have done this with out him. Thank you Dr. Gause for creating the masters program for military personnel in Molecular and Cellular Biology, and for the use of your lab instrumentation. I also thank you for going to bat for me on more than one occasion. You truly helped me get through some difficult times. I thank Capt David Kuch for paving the way as the first graduate of this program. David, thanks for your friendship and for showing me it could be done! To my fellow MCB class member, Robert Anthony, thanks for the laughs and companionship through all those seemingly endless classes and study group sessions. I wish you the best with your Ph.D. I also owe a debt of gratitude (and much more) to the United States Air Force, and Colonel Daniel Brown in particular, for giving me the opportunity and time to pursue this degree. I thank Dr. Joseph McCabe and Dr. Saibal Dey for their assistance as thesis committee members, Dr. Diana Cummings and Dr. Richard Mills for their assistance in the laboratory, and former professors, Dr. Farrar, Dr. Anderson, Dr. Cockayne for inspiration. Finally, there are a few others who deserve recognition for their assistance throughout the program, Dr. William Haffner, Dr. Jeffrey Harmon, Dr Cinda Helke, and Janet Anastasi, thank you all.

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CHAPTER I. INTRODUCTION

Significance

Estrogen is known to have a crucial role in the development, maintenance and differentiation of the female reproductive system. In addition, it has a significant role regulating non-reproductive systems such as the nervous and cardiovascular systems and bone density [1-4]. Moreover, evidence exists to suggest that estrogen may provide an important degree of protection from both neurodegenerative disorders and ischemic brain injury [5, 6]. Collectively, the physiologic role of estrogen is vital to the regulation of many different organ systems.

The study of estrogenic effects is important for a variety of reasons related to human health. First, prolonged exposure to endogenously occurring estrogen or synthetic estrogen-like compounds is linked to the development of cancer [8, 9] and has an important influence on the immune system [10]. In addition, women are living longer than before in the postmenopausal hypoestrogenic state; there are a growing number of studies to suggest that long hypoestrogenic state has a negative impact on a woman's health. Therefore, hormone replacement therapy (HRT) is being used to improve the health of aging women. On the other hand, amidst a great deal of publicity, and controversy, a portion of the National Institutes of Health Women's Health Initiative study was terminated due to concerns about a slightly increased risk of invasive breast cancer, coronary heart disease and stroke associated with a HRT regimen of estrogen and progestin [11]. The effects, both beneficial and detrimental, that arise from

HRT, are complex, and must be better understood. Gaining a complete understanding of estrogen physiology in the human body is at the heart of women's health.

Effects of Estrogen: Female Characteristics and Reproduction

Estrogen is not the only "female hormone" but it has historically been considered to be the major female sex hormone. Like other hormones, its presence in target tissues results in the growth and/or differentiation of specific cells and the effect can last for several hours or even days. Estrogen is responsible for a multitude of diverse biological effects in mammals. In females, it is responsible for differentiation, growth, and development of breasts, ovaries, and the reproductive tract. It plays a key role in regulating the menstrual cycle in humans and stimulates growth of the uterine wall in preparation of embryo implantation. Estrogens are also responsible for development of secondary female sex characteristics.

The Family of Estrogens

Estrogen is not a single hormone, but a family of hormones. While as many as six different estrogens are produced in humans, only three are produced in significant quantities. These are 17 beta-estradiol (E2), estrone, and estriol and are shown in Figure 1. Of these, E2 is the most effective compound and considered to be the major estrogen. It is 12 times more potent than estrone and 80 times as potent than estriol [12]. Because of the potency of E2 relative to the other estrogens, estradiol and estrogen are sometimes used interchangeably, at times mistakenly.

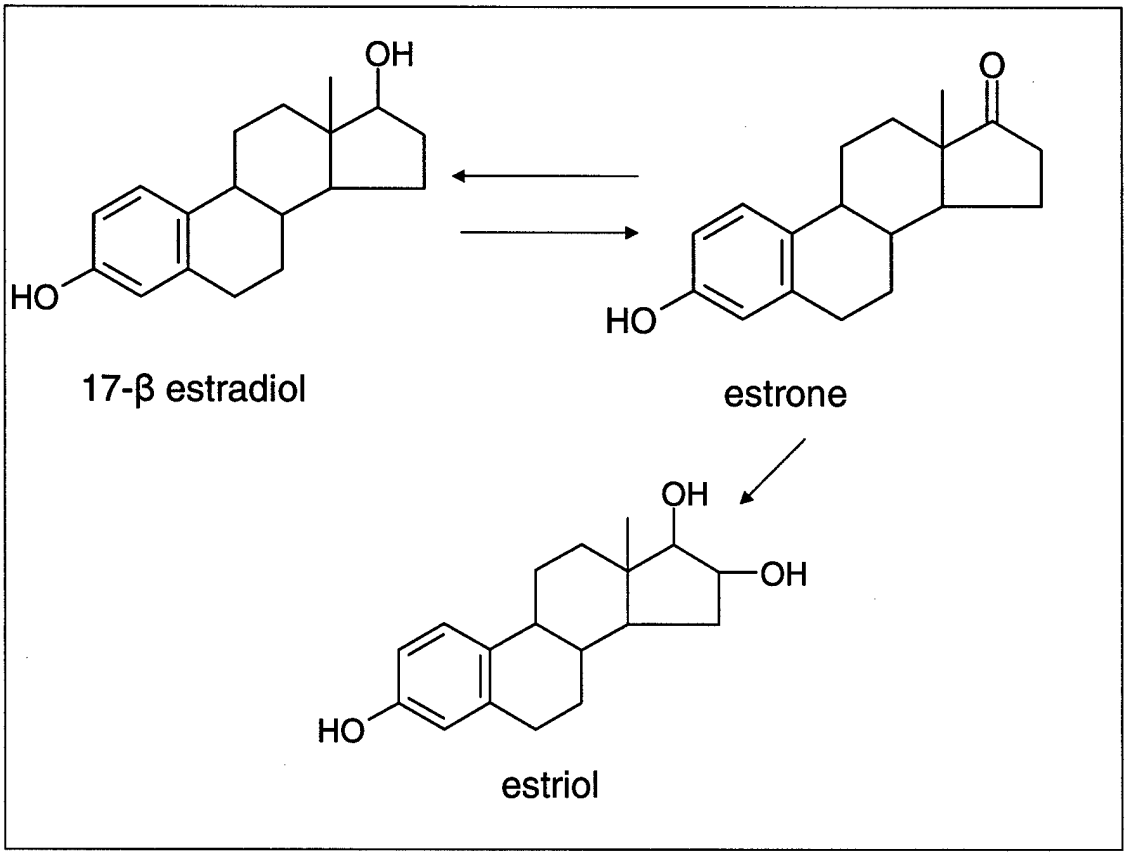

Figure 1. Structure of major estrogens. The three principle estrogens are 17 β-estradiol, estrone, and estriol. These are synthesized from cholesterol primarily in the theca and granulosa cells of the ovary, but are also produced in the placenta, adipose, and in much smaller amounts in other tissues.

Development of the Ovary

Estrogen is responsible for female characteristics. It is primarily produced and secreted by the ovary in non-pregnant females, though the adrenal cortices, adipose tissue, and some other tissues are capable of producing small amounts. Because the ovary is vital for estrogen production it is important to understand the development of this important organ.

Early during fetal growth, cells from the germinal ridge will develop into an undifferentiated gonad [12]. If the fetus lacks a functional Y chromosome, it will lack several genes responsible for male sexual differentiation, including the Testis Determination Factor (TDF) [13]. This factor, as the name implies, is responsible for causing the undifferentiated gonad to develop into a testis, which will, in turn, secrete androgens responsible for masculinization of the brain and reproductive tract. If TDF is absent, the undifferentiated gonad develops into an ovary capable of E2 production. It is this production of E2 by the ovary that is responsible for the development of primary and secondary female sexual characteristics and the ability for sexual reproduction.

Estrogen and the Onset of Puberty

From shortly after birth until puberty, the ovary produces very little E2. With the onset of puberty, and under the influence of pituitary hormones, E2 production increase by 20 times or more. Under the influence of this increased amount of E2, female secondary sexual characteristics rapidly develop such as the growth of the uterus, fallopian tubes, vagina, and external genitalia. An

increase in fat deposition in the mons pubis and labia majora occurs, and the labia minora increases in size as well. Under the influence of E2, the uterus increases two to three fold in size. E2 has an additional important effect on the uterus since it causes the endometrial stroma to develop and prepares it for implantation.

While E2 is not the only hormone responsible for breast function, it does cause the female breast to develop by causing an increase in fat deposits in the breast and regulating the development of stromal tissue and the extensive ductile system. Other hormones are responsible for complete development of the milk-producing function including differentiation of alveoli and breast lobules. E2 is further responsible for the modulation of the female pelvic structure. It also causes the epiphysial plates (or growth plates) in bones to fuse.

In summary, ovarian estrogen production vastly increases at puberty. E2 is responsible for the development of female reproductive organs as well as secondary female sexual characteristics.

Estrogen and The Female Reproductive Cycle

The human menstrual cycle is a 28-day cycle (on average), which continuously recurs during the childbearing years in women. At the beginning of the menstrual cycle, the endometrium is sloughed off and only a thin layer remains. Under the influence of increasing E2 levels in the second week of the cycle, the stromal cells and endometrial tissue proliferate and prepare the uterus for implantation of the fertilized egg. The level of E2 drops off shortly just before ovulation, and then increases again during the second half of the sexual cycle as

shown in Figure 2a. However, progesterone is the important hormone during this subsequent part of the cycle known as the secretory phase. Finally, just before the end of the cycle, the levels of E2 and progesterone drop off significantly and the cycle repeats itself if the fertilized egg is not implanted in the endometrium. Consequently, unlike androgen levels in males, which generally remain constant over time, estrogen levels vary from a high of approximately 300 pg/ml mid-cycle to a low of approximately 50 pg/ml in early follicular phase. This 28-day period of cyclical E2 is designated as the menstrual cycle.

Estrogen and progesterone are the ovarian hormones that are critical components in regulating the menstrual cycle. In turn, these ovarian hormones are regulated by the pituitary hormones: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Figure 2b displays levels of these pituitary hormones during the menstrual cycle. Finally, the release of LH and FSH from the anterior pituitary is under the influence of gonadotropin releasing hormone (GnRH) from a regulatory region of the brain known as the hypothalamus.

Estrogen and the Mouse Estrous Cycle

The female mouse reproductive cycle is referred to as the estrous cycle. The estrous cycle in the mouse lasts four or five days and consists of four phases: metestrus, diestrus, proestrus, and estrus (Figure 3). The estrous cycle is initiated post-puberty starting at 35-40 days after birth. Proestrus is the time during the cycle when the follicles are growing. The growing follicles produce E2, thereby increasing levels of E2 in the bloodstream. This results in the LH surge, which brings on estrus. During estrus, the female is receptive to the male and is

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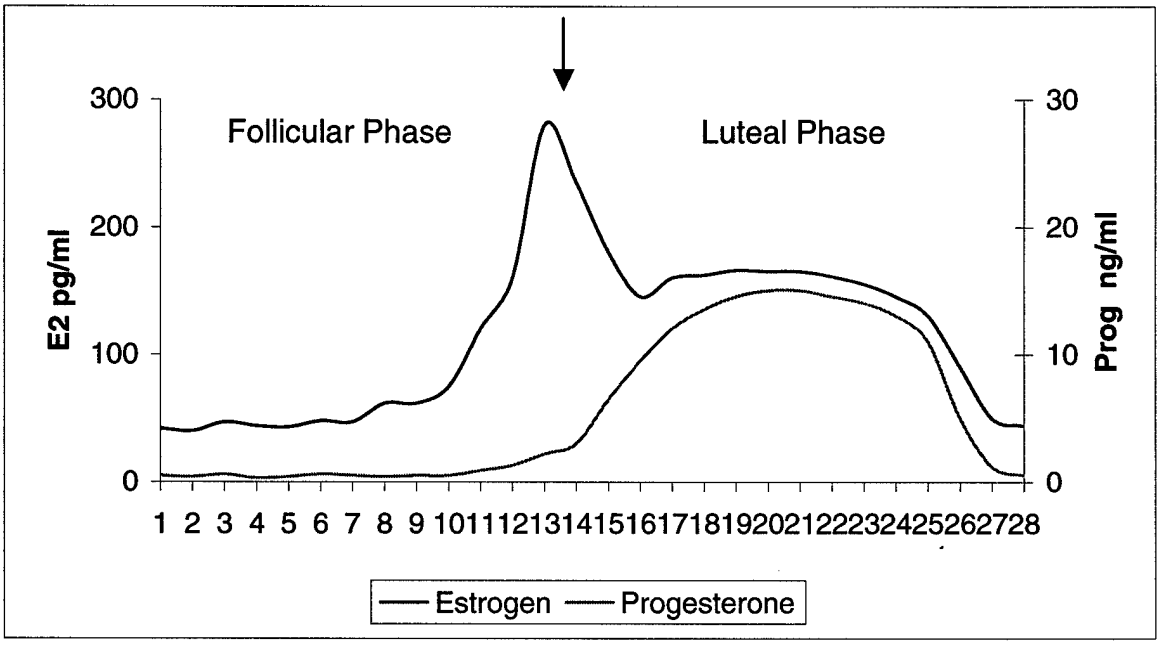

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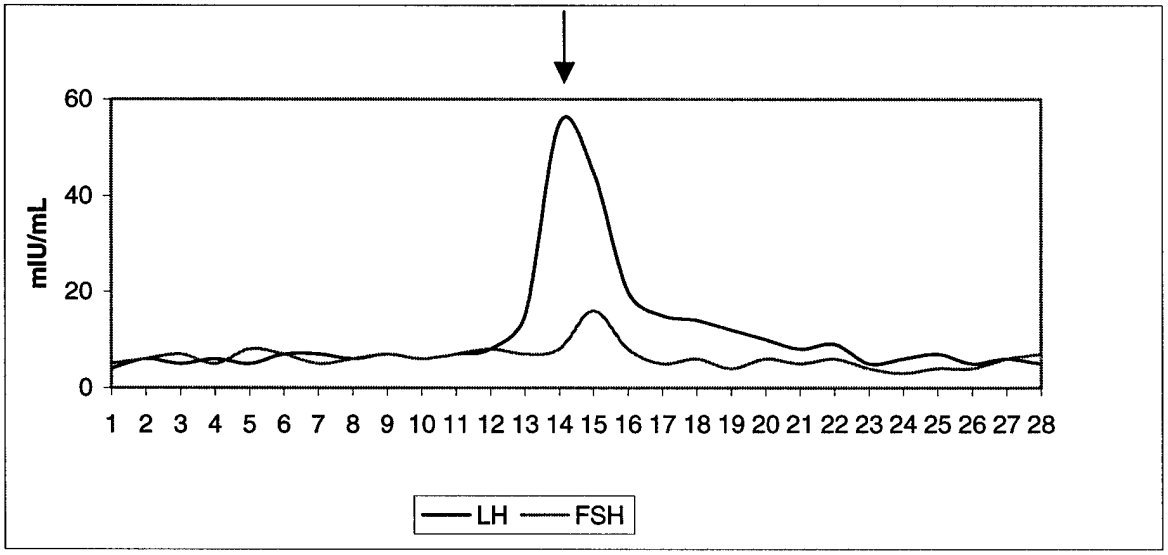


Figure 2. Hormone profile during the menstrual cycle. A. Changes in estrogen and progesterone. Estrogen levels peak just prior to ovulation (arrow). Progesterone levels remain low during the follicular phase then rise during luteal phase. B. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels during the menstrual cycle. Both hormones surge prior to ovulation (arrow) causing the follicle to rupture and the ovum to be released from the ovary.

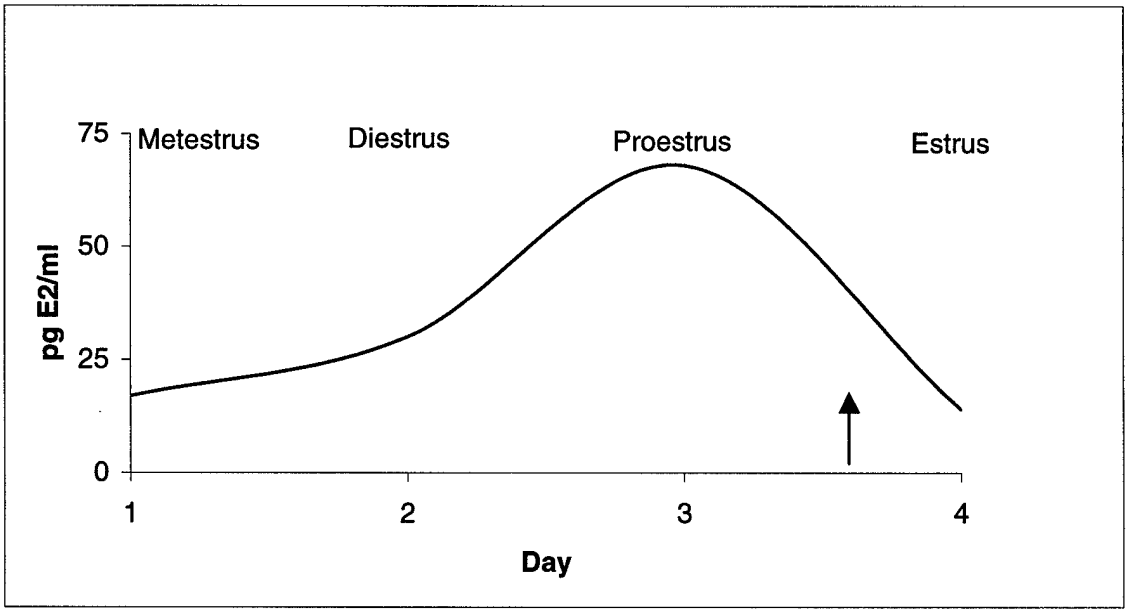


Figure 3. Estradiol levels during the mouse estrous cycle. E2 Levels are low during metestrus, begin to rise at diestrus before reaching their peak during proestrus then fall again at estrus. The rise during proestrus primes the nervous and reproductive systems to induce behavioral estrus and prepare for ovulation (arrow), respectively.

ready to ovulate. After ovulation, the corpora lutea develop from the disrupted follicles. This is the period known as metestrus. Diestrus is the period when the corpora lutea secrete progesterone.

Estrogen Regulation: The Hypothalamus-Pituitary-Gonad Axis

The hypothalamus is the principal region of the brain responsible for regulating female reproduction. Here, neurons are subject to feedback regulation from the physiological processes they control. Some cells of the hypothalamus release either hormones or neural signals to the pituitary, which, in turn, releases gonadotropic hormones into the bloodstream to act on the gonads. These, in turn, manufacture and release hormones, that will eventually feedback to the hypothalamus as shown in Figure 4. With respect to estrogens, the hypothalamus secretes GnRH, which acts on the pituitary to release LH and FSH. These hormones then stimulate the theca and granulosa cells in the ovary to produce E2, which circulate in the bloodstream and act on target tissues. Neurons in the hypothalamus are able to detect plasma levels of estrogen and then respond to regulate them as the cycle continues. This axis is responsible for controlling the menstrual cycle in humans and the estrous cycle in rodents. As women age and near the "menopause" period, the hypothalamus becomes less responsive to estrogens, resulting in disruption of the normal cycle [6]. The release of GnRH is delayed, which in turn, delays the surge of LH release from the anterior pituitary. This asynchrony is responsible for onset of irregular cycles and eventually leads to menopause [6, 14].

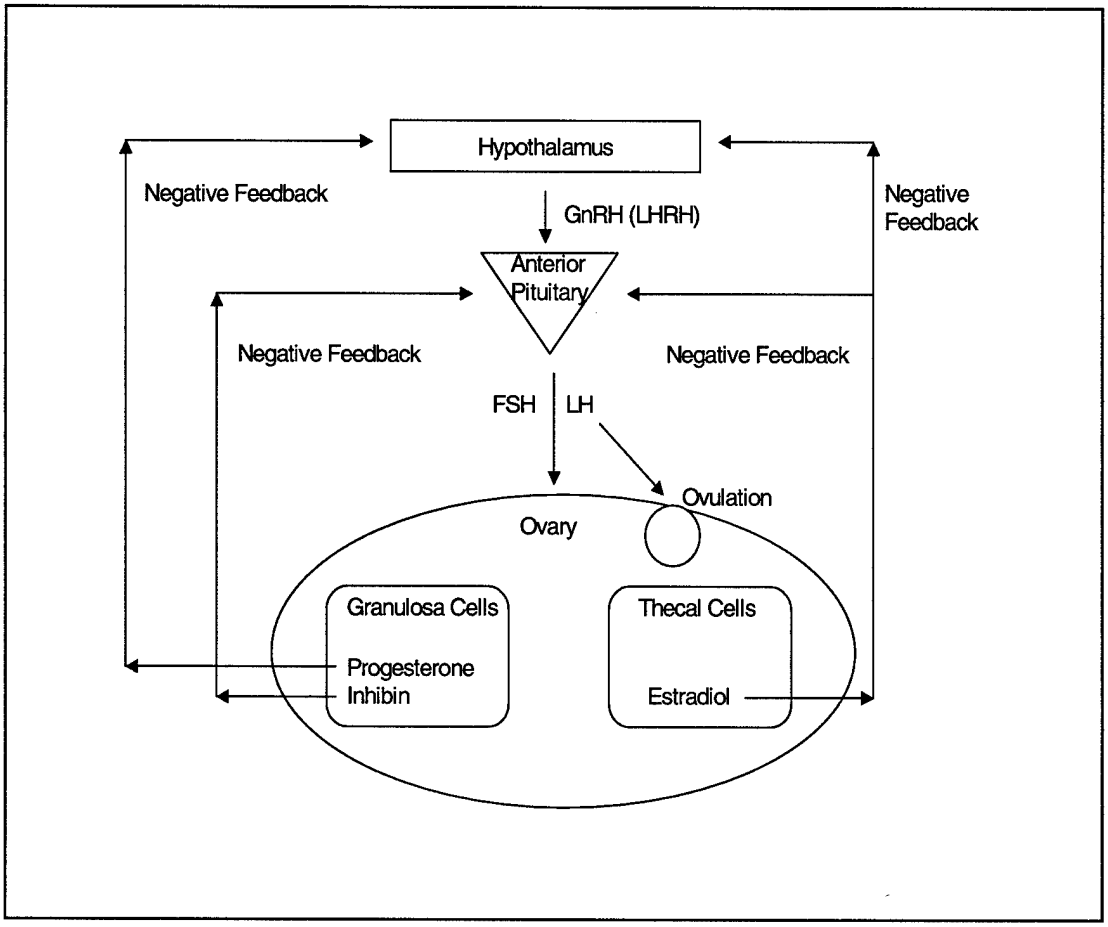


Figure 4. Three axes of reproduction: hypothalamus, pituitary, and gonad. Sex hormone levels are regulated by the hypothalamus where GnRH is secreted. This hormone acts on the anterior pituitary, which releases follicle stimulating hormone (FSH) and luteinizing hormone (LH) into the bloodstream where they act on the gonads. Under the influence of pituitary hormones, the ovary releases estrogens and progesterone (sex hormones) into the circulatory system. The hypothalamus senses them and decreases GnRH secretion in a negative feedback loop in humans. Negative feedback is also mediated by inhibin, a regulatory hormone secreted by the ovary.

Non-Reproductive Effects of Estrogen

While the most well known effects of estrogen are related to development of female characteristics and reproduction, studies continue to provide evidence of additional beneficial effects mediated by this hormone. Some of these include bone metabolism, cardiovascular health, and skin tone.

For some time it has been understood that E2 is a major influence in regulation of bone metabolism in females and that hypoestrogenic, post-menopausal women are at increased risk for osteoporosis [1]. However, new studies suggest that estrogen plays an important role in bone density of men as well [2]. The enzyme cytochrome P450 aromatase converts the sex-steroid testosterone to estradiol. Men with mutations in the aromatase gene have bone density problems such as osteopenia and osteoporosis [15].

Estrogen's constructive influence of the heart is well documented. Catechol estrogens have been shown to improve cardiovascular health while decreasing the risk of cardiovascular disease [3]. Estrogen activates endothelial nitric oxide synthetase, which leads to vasodilation. Moreover, E2 seems to indirectly promote decreased serum triglyceride levels and increased serum HDL levels. This results in a good serum lipid profile, which leads to a decreased risk of heart disease. Furthermore, estrogen affects coagulation and fibrinolytic proteins in a way that reduces hypercoagulopathies [4]. All of these effects add up to an overall positive influence on cardiovascular health.

In addition to the benefits to bone and heart, estrogens have an important effect on the skin as well. They appear to be important regulators of the

connective tissues hyaluronic acid and collagen and consequently increase skin thickness [7, 16]. They have been shown to be effective in stimulating repair of skin damaged by UV light. In this study, ovariectomized rats were observed to have problems with skin aging faster than normal rats due to unrepaired UV damage [17].

There is a growing body of evidence suggesting estrogen plays a significant role in learning, cognition, mood, and memory [18]. Several estrogenic effects may be responsible, including increased cerebral circulation, improved immune response, mediation of important neurotransmitters and hormones, anti-apoptotic influence, anti-inflammatory actions, and anti-oxidant properties [19]. Other studies provide evidence suggesting estrogen may modulate specific cognitive functions such as working memory and verbal learning and memory [20]. Ovarian steroids have widespread effects throughout the brain serotonin pathways, catecholaminergic neurons, and the basal forebrain cholinergic system as well as the hippocampal formation. Postmenopausal women on estrogen replacement therapy did much better than their counterparts in a study that evaluated short-term and long-term memory and the ability to learn new associations [21].

Alzheimer's disease (AD) is a malady primarily of the elderly that affects the brain and causes dementia. It is believed the pathogenesis of this ailment is primarily the result of deposition of amyloid beta protein, which, in turn, results in neurofibrillary tangles and other pathogeneses [22, 23]. Post-menopausal women who are not on HRT are two to three times as likely to develop AD than

men their same age [5]. The literature has limited data on the effectiveness of estrogen on women who already have AD [24], but those women who are taking HRT may be at a lower risk of developing AD than those who are not [24, 25]. Furthermore, the onset of the disease is delayed in women on ERT [26], [27].

Ischemia is defined as the damage that occurs to cells and tissues due to a lack of blood flow, which causes oxygen starvation and toxicity from waste buildup. Ischemia is usually caused by a blockage in a blood vessel. When this happens in the brain the result is a stroke. New evidence suggests estrogen provides a neuroprotective effect against this kind of brain injury [6]. For example, ovariectomized rats given physiologic levels of E2 prior to an ischemia-like trauma fared much better than those not receiving estrogen [6]. One possible explanation is that the neuroprotective effect results from decreased levels of apoptosis due to interactions with the bcl-2 family of proteins [25]. Interestingly, rats that received estrogen antagonists in conjunction with estrogen resulted in greater damage to the brain [28]. This would suggest that estrogen's neuroprotective effect only comes from the binding of E2 with the estrogen receptor and resulting downstream effects.

In summary, estrogen has been thought of as the female sex hormone responsible for female characteristics and reproduction. It is important to appreciate, though, that estrogen is a hormone of great significance involved in the regulation of many important physiological processes in both females and males. It is beneficial to discuss how estrogen causes these physiologic effects at a cellular level.

Steroid Hormone Action

Mammals are complex multi-organ creatures. Communication between cells, tissues, and organs is vital in safeguarding the homeostasis of the organism. Cells communicate with each other through various mechanisms. One way in which extra cellular signaling occurs is via secreted molecules. This type of cell-cell communication has been further classified into three general categories known as paracrine, autocrine, and endocrine signaling. The distance the signaling molecule actually travels differentiates these three types of signaling. Autocrine signaling occurs when a cell secretes a signaling molecule that is picked up and bound by its own receptors. Paracrine signaling occurs when a cell secretes a chemical signal that binds to receptors on local or adjacent cells. Finally, endocrine signaling includes the secretion of hormones, which are released into the blood stream and are carried some distance to act on target cells or tissues, sometimes at great distances. In humans, the endocrine system is complex and includes many tissues and signaling molecules. The principal estrogen, E2, is synthesized in many tissues [29] but in non-pregnant woman and in men, it is primarily synthesized in the ovaries and testes, respectively. The key regulator of estrogen circulation and concentration is the HPG discussed earlier.

A variety of molecules act in the signaling mechanisms described above. Some of these include, neurotransmitters, amino acid derivatives, small peptides, cytokines, hormones, and small molecules such as nitric oxide. The several different classes of hormones are based on composition and location of

receptors. The hormone may either be lipid or peptide-based. Steroid hormones are synthesized from cholesterol and are able to diffuse across plasma membranes and bind to intracellular receptors. Steroid hormones are further divided by function into reproductive steroids, mineralocorticoids, and glucocorticoids. These latter groups have various functions including initiating the "fight or flight" response and regulating water retention in the kidneys and blood pressure. Other lipid hormones such as the prostaglandins or leukotrienes are known as the eicosanoids. Receptors for this type of lipid hormone are located on the cell surface. These are derived from an essential polyunsaturated arachadonic acid precursor and have a variety of functions including assisting in coagulation and inflammation. Finally, peptide hormones are derived from amino acids and bind to cell surface protein receptors. Examples include glucagon and insulin, both of which are involved in the regulation of plasma glucose levels.

The family of sex steroid hormones includes the androgens, the progestins, and the estrogens. These hormones are responsible for initiating the development and differentiation of the male and female reproductive systems and for the masculinization of the male brain or feminization of the female brain. They also regulate reproductive processes and behaviors in adults. The effects of estrogen were well documented above.

The primary progestin is progesterone. Shown in Figure 5, progesterone is a female hormone responsible for certain effects in the menstrual cycle and suppressing ovulation during pregnancy. It is also important in preparing the mucous layer of the uterus for implantation of the ovum. In addition,

progesterone plays a supportive role in milk production and secretion in the lactating breast. Furthermore, some believe it prevents cognitive dysfunction as part of HRT in post-menopausal hypoestrogenic women.

The principal androgen is testosterone (Figure 5). This hormone is responsible for male sex characteristics including increased muscle mass, deepening of the voice, growth of body hair, development of the male gonads, and other secondary male characteristics. Testosterone is also an important precursor to estrogen, which is synthesized from testosterone by cytochrome p450 aromatase.

Though estrogen has been considered the major female reproductive hormone, it is also important in regulating male reproduction. Too much estrogen in males hinders spermatogenesis, and the complete lack of estrogen (caused by cytochrome p450 aromatase deficiency) leads to sterility in men [30, 31]. A correct ratio of testosterone and estrogen is important in spermatogenesis. In addition to spermatogenesis, estrogens can also exert a negative influence on the developing male reproductive system and may even be the cause of certain developmental abnormalities [32]. Moreover, previous studies show that estrogen plays a pivotal role in serum concentrations of GnRH in males, and may even influence male sexual behavior [33].

Because they are synthesized from cholesterol and are lipophilic, the sex steroids bind to receptors inside the cell. These steroid nuclear receptors are discussed in greater detail here.

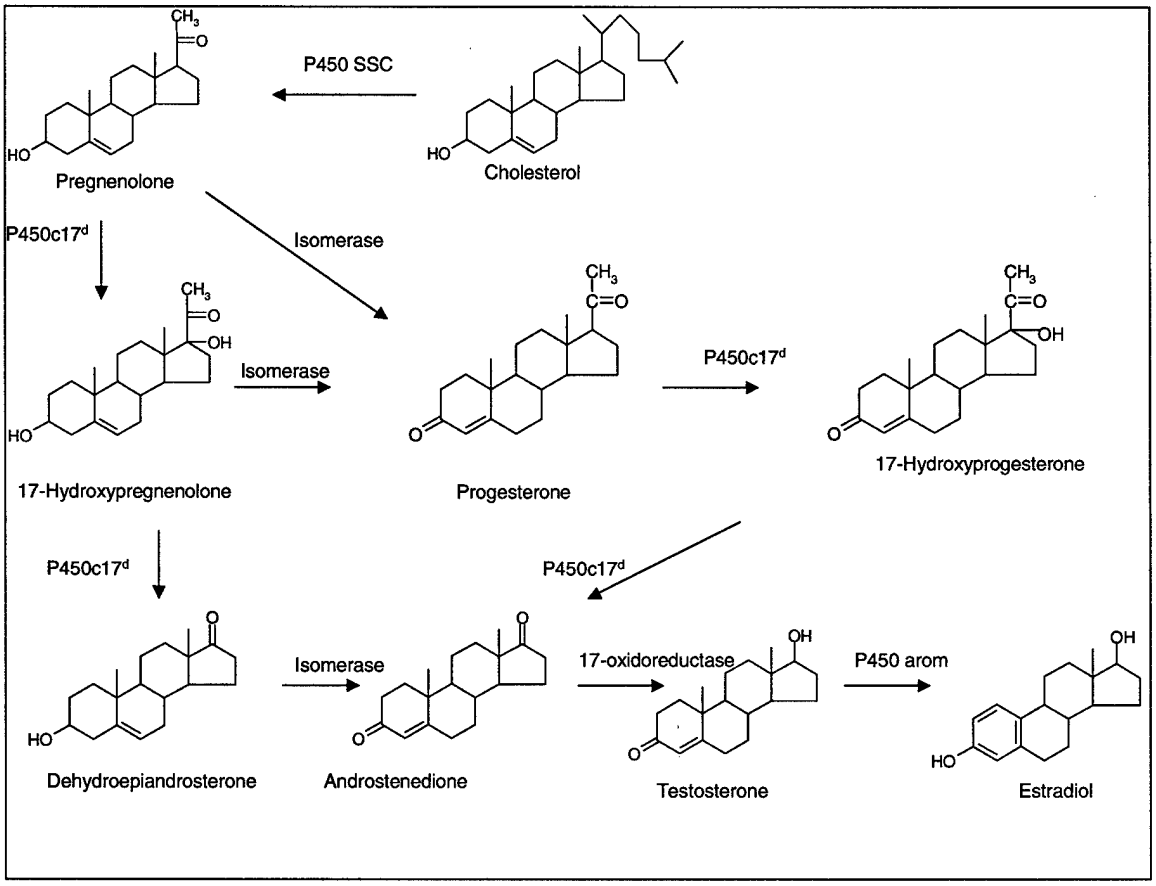


Figure 5. Steroid metabolism pathways. Cholesterol is converted to progesterone, testosterone and estradiol through various chemical reactions, each catalyzed by a specific enzyme.

Steroid Nuclear Receptors

In order for estrogen to affect target tissues, they must express the estrogen receptor (ER). In order for estrogenic effects to occur, estrogen must first bind with its receptor. As a synthetic product of cholesterol, estrogen is lipophilic and readily diffuses across the lipid bilayer of cell membranes. When estrogen binds to its receptor, events take place that lead to changes in levels of transcription or other cellular responses. Consequently, it is expected that estrogen responsive tissues would express ER, and non-responsive tissues would have low to no expression of this transcription factor.

The ER belongs to the steroid nuclear hormone receptor superfamily of proteins. At the cellular level, estrogen regulates transcription of specific genes containing the estrogen response element (ERE), an upstream regulatory sequence. Nuclear hormone receptors are proteins that contain several important functional domains including the important DNA binding domain and the activation domain. The DNA binding domain is fairly conserved throughout this family of receptors. When bound to its ligand, the receptor undergoes conformational changes that allows it to dimerize and bind to DNA at a sequence known as the Hormone Response Element (HRE) and modulate transcription as shown in the diagram in Figure 6. The DNA sequences situated just upstream of the initiation sites of many genes has important regulatory functions. A complex comprised of dimerized hormone receptors, together with their associated cofactors, modulates transcriptional activity of HRE-regulated genes. When the

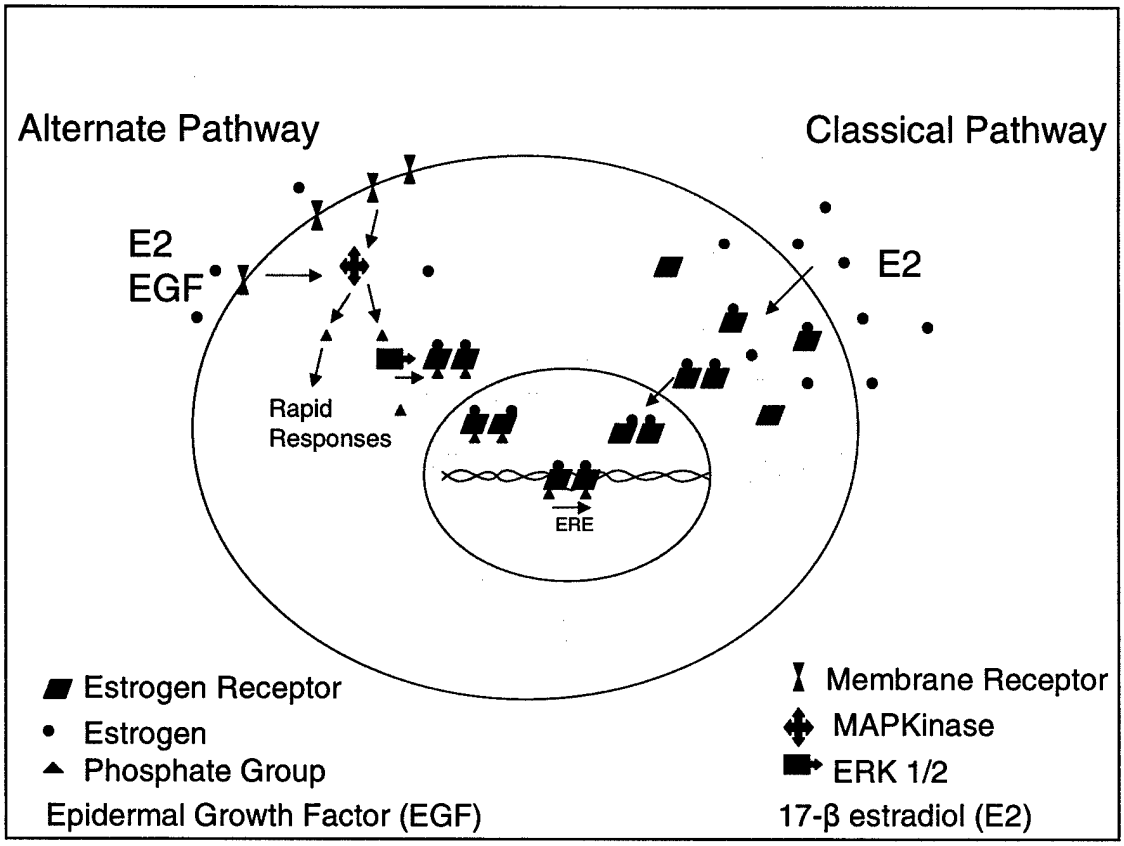


Figure 6. Classical and alternate estrogen receptor (ER) signaling pathways. In the classical pathway, estrogen diffuses to the nucleus and binds to ER to regulate transcription. The alternate pathway is not well understood, though it is believed estrogenic effects are mediated by membrane-bound ERs, resulting in activation of signal transduction cascades and second messenger systems.

ligand-bound receptors bind to the DNA at the HRE, a cascade of events takes place, which results in either increased or decreased transcription and corresponding level of protein product [34]. The steroid nuclear receptor family consists of the ER, progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). The genomic mechanisms of action are similar with all of the steroid nuclear receptors but the focus here is the ER.

Classical and Alternate Signaling Pathways

The ER is a 595 amino acid protein. The two significant domains of this protein are its ligand-binding domain (LBD) and its DNA-binding domain (DBD). Figure 7 provides an illustration of the ER. This receptor is believed to have two general mechanisms of action [35]. The first of these is known as the classical, or genomic pathway. In this pathway, the lipophilic estrogen compound diffuses across the plasma membrane and interacts with estrogen receptors in the cytoplasm. Bound to ligand, ER dimerizes and translocates to the nucleus where the resulting hormone-receptor complex binds to the DNA at the estrogen response element (ERE), a transcription control region, and affects expression of specific genes. The effects of E2 on growth or differentiation in this pathway often last for hours or days. Interestingly, some of the effects caused by estrogen seem to take place too rapidly (<30 minutes) to be explained by the classical pathway. Because of this, non-classical signaling pathways have been proposed [32, 36-38]. With this non-genomic pathway, estrogens bind to receptors on or near the cell membrane and kinase-signaling pathways are

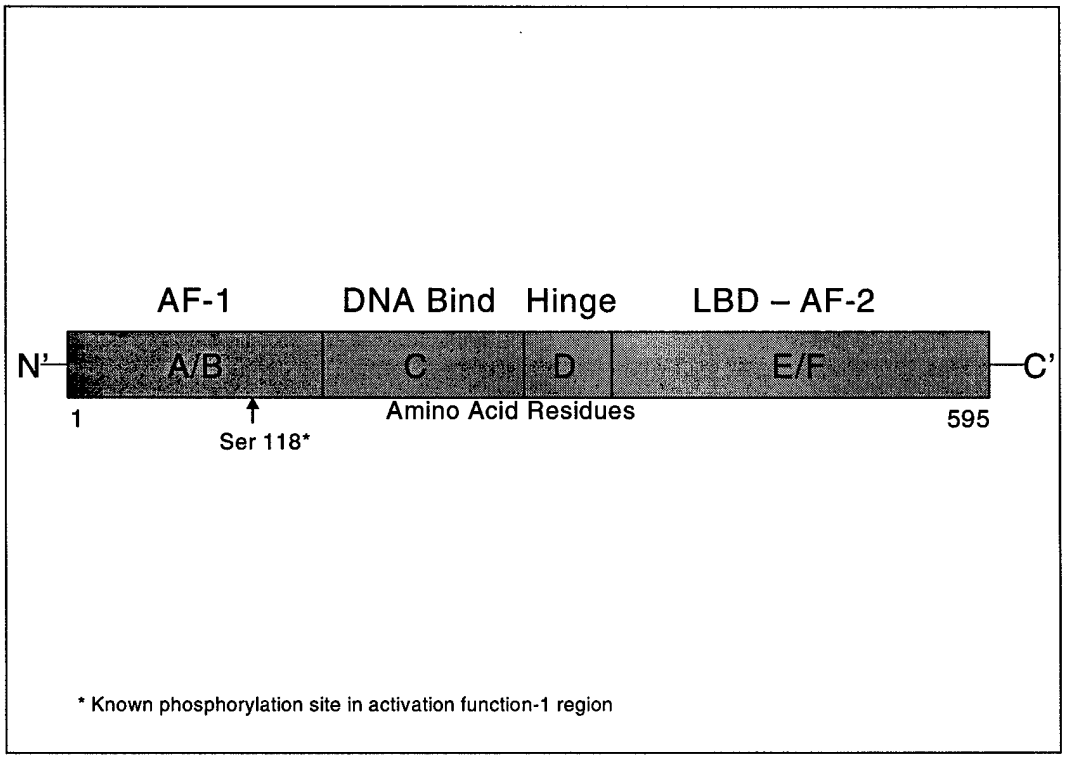


Figure 7. The structure of estrogen receptor. The A/B region contains the activation function-1 site. Here, the ER may be activated through phosphorylation of residues, such as the known phosphorylation site at serine 118. Region C contains two zinc-finger motifs, responsible for binding to the DNA molecule. The E/F domain contains the residues involved in ligand binding.

activated (Figure 6).

This pathway is believed to be responsible for estrogen's effect on levels of second messenger molecules such as cyclic AMP (cAMP) and the calcium ion (Ca^{++}), and activation of mitogen activated protein kinase (MAPK) pathways. Additionally, it is known that activation of ER results in increased phosphorylation in the cell and that ER itself can be phosphorylated, causing an increase in its effectiveness.

The Isoforms of ER

For many years it was thought estrogen-responsive tissue expressed a single type of estrogen receptor. However, a second receptor was discovered in 1996 in prostate and ovary tissues [39]. This additional receptor was designated ER-β, while the original ER was designated ER-α. It appears ER-α and ER-β share a high degree of homology and both regulate estrogen responses [40]. Since ER- β was discovered in 1996, additional studies have led to the discovery of possibly even more isotypes [38, 41]. Interestingly, studies show that in estrogen-responsive tissues, cellular expression of ER-α and ER- β is quite varied. In the female reproductive tract, for example, expression of ER-α seems to predominate in the uterus, vagina and oviduct, while ER- β predominates in the ovary [42, 43, 44].

In the brain, there is some overlap in the distribution of the two receptor isotypes. However, certain regions express only ER-α or ER-β. In the rodent brain, both ER-α and ER-β mRNA transcripts and protein have been isolated [43, 45]. This may provide an important redundancy for crucial neuroregulatory

functions. ER-α knock-out mice are still able to respond to E2 and demonstrate estrogenic effects in the brain. Interestingly, ER-α knock-out male mice are infertile but ER-β mice are not [46].

In the pituitary, both ER-α and ER-β have also been found, although the ER-α is seen more abundantly [45, 47]. In the anterior pituitary, both types of receptors are found in lactotrophs, corticotrophs, follicular-stellate cells, melanotrophs, and gonadotrophs. But less than 50% of the cells of each of these cell types expressed either ER. In melanotrophs and gonadotrophs, ER-β was present but expressed in significantly lower amounts than ER-α. Furthermore, of the pituitary cells that expressed estrogen receptor, only 6-10% of cells expressed both isotypes together [47]. Additional studies are needed to better understand the different regulatory processes moderated by ER-α and ER-β in the brain and pituitary.

Proto-Oncoprotein Brx

The link between estrogen and cancer is well known. Hormones play a key role in differentiation and development of several cancers including cancers of the breast and endometrium [48, 49]. In the year 2001, approximately 625,000 women in the United States were diagnosed with cancer [50]. Of these women, breast cancer was diagnosed most frequently compared to other forms of cancer (25-30%). Similar to other cancers, breast cancer is a disease characterized by the uncontrollable growth and spread of abnormal cells. Consequently, much research has been done to better understand factors affecting cell growth and cycling. Through this research much has been learned about proteins known as

growth factors, tumor suppressors, and proto-oncoproteins. These are the major regulatory proteins of cell growth, differentiation and cycling and it is the mutation of these genes that ultimately leads to the development of cancer [51].

Growth factors bind to receptors either on the cell membrane or, in some cases (steroid receptors, for instance), within the cell itself. When growth factors bind to their respective receptors, they initiate conformational changes that set off signal cascades. These signals lead to growth, differentiation, and division mediated by phosphorylation cascades, second messengers (cyclic AMP and calcium), and activated transcription factors. Growth factors may be peptides, steroid hormones, cytokines, or other activating substances. Sometimes growth factor receptors are mutated in such a way that allows them to bind non-specifically to many substances other than the intended factor. When this happens, the cell becomes overly stimulated [52]. Alternatively, overstimulation can be due to an abnormal increase in the production of a particular growth factor. This may be due to an endocrine system malfunction or over production of local factors such as tumor growth factor, or due to environmental chemical exposure that simulates growth factors [48]. Regardless of the cause, excessively stimulated cells may lead to cancer [53, 54].

Tumor suppressor genes have different specific functions, but in general they put a halt on the cell cycle when the DNA has been damaged. Cells have their own built-in repair mechanisms including DNA replication [55]. When damage occurs to DNA cells attempt to fix the problem [56]. If the damage is irreparable it takes advantage of the tumor suppressor genes [57]. A well-

studied tumor suppressor protein found mutated in many different cancers is p53 [58]. When a mutated p53 is incapable of halting the cell cycle of damaged cells, the daughter cells receive an incorrect copy of DNA. This leads to the propagation of mutations in subsequent cell divisions. The acquisition of such genetic abnormalities leads to cancer.

Proto-oncogenes are the regulatory proteins of cells. These proteins become activated through a variety of signals, and when they do, they induce the cell to increase transcription. One well-known proto-oncoprotein important in multiple cell signaling pathways is a protein called Ras. This proto-oncoprotein is a protein that acts like a switch that can be turned on or off; it cycles between inactive and active states [59]. When Ras is bound to a substance called guanine diphosphate (GDP) it is inactive. However, when GDP is replaced by guanine triphosphate (GTP) it becomes active. This occurs as part of a signaling cascade initiated by a growth factor. Unfortunately, however, proto-oncogenes become oncogenes (or cancerous) when they are mutated in such a way that causes them to function continuously in the active state [60, 61]. It is as if the "switch" is stuck in the on position. The cell simply proliferates unregulated and becomes cancerous.

In the early 1990's researchers were looking at proteins involved in the inhibition of estrogen responsive genes and discovered an auxiliary protein that was associated with Retinoid X Receptor (RXR) inhibition [62, 63]. The RXR is a non-steroid orphan nuclear receptor that binds to sites with 5'-AGGTCA-3' sequence on DNA and forms heterodimers with a variety of partners in the

nuclear receptor family to regulate transcription [64]. Additional studies were done to identify the auxiliary protein binding to RXR. Using a breast cancer expression library, researchers studied the proteins that bound to RXR and discovered a novel cDNA and cloned it [65]. This group learned the new protein bound to nuclear hormone receptors and was similar to a known cell-signaling protein called Lbc [66]. They called the protein Brx since it was found in breast tissue, it bound to nuclear receptors, and was an auxiliary protein [65].

The function of any protein may be determined by identifying its role in a biochemical process or by doing a search to identify similarities to other proteins with known function. Both methods were used with Brx. Initially, it was discovered that Brx shared similar regions with the cell signaling proteins (proto-oncogenes) Rho and Lbc [65]. These proteins contain a functional region known as a guanine nucleotide exchange factor (GEF). The GEF is responsible for mediating signaling of proteins such as Ras, mentioned above. In the case of Lbc it was found to bind to a signaling protein, Rho [66]. Brx and Lbc (along with other proteins in this Dbl family) are important in guanine nucleotide exchange and help activate Ras-like proteins as they change cycle between the active and inactive state based upon whether they are bound to GDP or GTP [59, 67]. *In vivo* interaction studies were done to biochemically demonstrate the function of Brx in cell-signaling regulation. It was determined that Brx regulates activity of ER-α through a MAPK Cdc42 dependent pathway [65] and ER-β through a p38 MAPK pathway [68].

A sequence homology analysis showed that Brx contains several significant functional regions [65] as illustrated in Figure 8. The N-terminal region has some homology to an AKAP protein designated Ht-31 [69-71]. The second region contains a diacylglycerol (DAG) binding site. DAG is a second messenger involved in cell signaling pathways and cytoskeletal reorganization [72-74]. The third region contains an EF hands motif, which is known for the helix-loop-helix structure and its ability to bind the calcium ion and change the conformation of a protein to its active state [75, 76]. The next region of interest contains the tandem Dbl-homology oncology domain (DH) and the Plekstrin homology domain (PH). These domains are seen in the Dbl family of proto-oncogenes and GEF's. The DH domain is the catalyst for GEF nucleotide exchange and the precise function of the PH domain is not well known but since the region binds to phosphoinositides it is thought to help localize the action of proteins with this domain [67 59, 77, 78, 79]. The C-terminus contains a novel region that binds nuclear hormone receptors. While Brx has been shown to bind to other nuclear hormone receptors such as RXR, thyroid hormone receptor (THR), and peroxisome proliferator-activated receptor (PPAR), it seems to bind with highest affinity with the estrogen receptor [65]. Consequently, we expect to find Brx in tissues that express the ER.

Specific Aims

Brx is known to enhance ER-regulated transcriptional activity. The association of Brx with the estrogen receptor led us to hypothesize that it may be found in estrogen responsive tissue in the CNS. The goal of this thesis was to

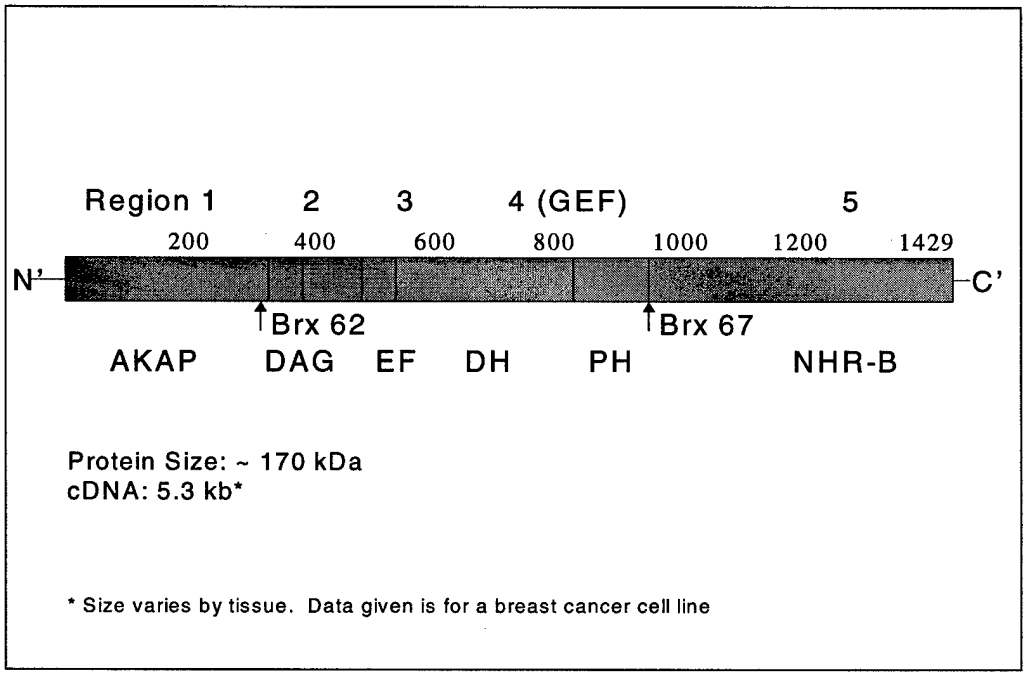


Figure 8. The structure of Brx. The AKAP region binds cyclic AMP and tethers the protein. DAG and EF hand motifs may be involved in modulating protein kinase C. The DH and PH domains are responsible for the guanine nucletide exhange properties of the protein. The c-terminal region binds nuclear hormone receptors.

determine the localization of Brx in the adult mouse brain and pituitary. The specific aims were the following: A) To determine the localization of Brx in the brain and pituitary. Brain and pituitary contain significant levels of estrogen receptors. The aim was to use immunocytochemistry (ICC) to characterize patterns of Brx protein in brain and pituitary of adult male and female mice. B) To compare levels of Brx expression in brain and pituitary of males and females. Because estrogen levels vary significantly between males and females, it was felt Brx expression may be sexually dimorphic. This specific aim was to determine if there were differences in Brx protein expression in brain and pituitary of adult male and female mice. C) To determine the ability of estrogen to regulate Brx expression in the adult mouse brain and pituitary. This aim was to determine if ovarian steroids were involved in the regulation of Brx in the ovariectomized, adult female mouse.

CHAPTER II. MATERIALS AND METHODS

The questions in this thesis were investigated using a mouse model. The approach taken to address the specific aims includes the use of two methods, immunocytochemistry (ICC) and Western blot.

Tissue Preparation

Animal Care

Male and female C3H mice (5-8 weeks of age) were purchased from Taconic Farms (Germantown, New York) and housed under controlled conditions of light (12:12 lights on at 0600h) and temperature (22-25° C). Food and water were provided to the animals *ad libitum*. All animal procedures and care were conducted in accordance with the standards approved by the NIH Guide for the Care and Use of Laboratory Animals.

Perfusion

Animals were acclimated for at least one hour before each procedure. All animals were killed between 1000 and 1200h. The mice were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg BW, i.p., Abbot Labs; North Chicago, IL) and perfused intraventricularly with 5-10 ml cold saline (0.9% NaCl) followed by 50 ml fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3). The brain and pituitary were post-fixed overnight in the same fixative, and then embedded in gelatin followed by saturation in graded sucrose solutions at 4° C (10% sucrose in 0.1 M PB overnight followed by 20% sucrose in 0.1 M PB overnight). After sinking in 20%

sucrose solution, the embedded brain blocks were quick frozen in ice-cold isopentane (-70° C) and stored at -80° C storage until cryosectioned. Pituitaries were cryoprotected as described above, but were not embedded in gelatin. They were embedded in OCT and quick-frozen in isopentane over dry ice for storage at -80° C.

Microtomy

Brain and pituitary samples were equilibrated to -20° C and subsequently to –24 ° C for one hour prior to cryosectioning. Coronal sections (40 µm) were cut rostral to causal from the olfactory bulb to the midbrain/cerebellum and collected in ice-cold Tris-Buffered Saline (TBS; 0.1 M, pH 7.2). All tissue sections were stored at 4° C until ICC. In contrast to the brain, 10-µm thick sections were cut from pituitaries and immediately mounted onto glass slides. Slides were stored at –20° C storage until experimentation.

Fresh Frozen Tissue Collection

Male and female mice were sacrificed by lethal inhalation of CO_2 followed by decapitation. The brain and pituitary were rapidly removed from the calvarium and dissected. Tissue sections including the cortex, thalamus, hypothalamus, olfactory bulbs, cerebellum were dissected and frozen on dry ice within four minutes of decapitation. In addition to the CNS, heart, spleen, liver, uterus, lung, and the gonad were collected as controls. All samples were stored at –20° C and extracted for protein with one week.

Ovariectomies

Animals were anesthetized with a mixture of ketamine/xylazine (i.p.;100 mg/kg BW ketamine; Ketaset, Animal Health, Fort Dodge, IA and 10 mg/kg BW xylazine, Xyla-Ject, Phoenix Pharmaceutical, St. Joseph, MO,). After anesthesia, the animals were shaved on their dorsal trunk, and then laid on their ventral surfaces. The area was draped, and sterilized with betadine and alcohol. A small midline incision was made through the skin approximately half way between the shoulders and the rump. Incisions were then made through muscle to gain access to the peritoneal cavity. The ovaries were excised and removed. After ovariectomy, the animals were allowed to recover for one week prior to experimentation.

Estrogen Treatment

To test the effect of estrogen on Brx expression levels, the ovariectomized mice were randomly assigned to receive either vehicle (oil) or 20 μg 17-β estradiol benzoate (Sigma, St. Louis, MO), subcutaneously. The mice were injected between 1000 and 1200h. The control animals received injections of oil vehicle. The animals were killed and tissue collected 48h after treatment.

Immunocytochemistry

Single Label Free Floating ICC

Brain sections were processed by free-floating ICC. Sections were rinsed twice in 0.05 M TBS for five minutes each prior to incubation in 1% sodium borohydride (Sigma) in TBS for 20 minutes. This incubation was followed by 1% hydrogen peroxide in TBS for 10 minutes to inactivate endogenous peroxidase activity. The sections were sequentially incubated in a blocking solution

containing 0.1% bovine serum albumin (BSA; Sigma), 5% normal goat sera (NGS; Vector Laboratories, Burlinghame, CA) and 0.5% Triton X-100 (EM Science, Gibbstown, NJ) in TBS for 1hr, a primary antibody to Brx (1:1000 in 0.05 M TBS plus 0.5% NGS and 0.05% Triton X-100; a gift from Dr. James Segars) for 48h at 4° C, biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratory, Burlingham, CA), followed an avidin-biotin-horseradish peroxidase complex (1:400; Vector) for 1.5h. Four 5 minutes washes were performed between each incubation. Immunoreactivity was visualized with 3,3' – diaminobenzidine tetrahydrochloride (ICN Biomedicals, Aurora, OH) in TBS. Following extensive washes in TBS, sections were mounted onto microscope slides. Sections were allowed to dry overnight, rinsed in deionized water, dehydrated in alcohols and cleared in xylene, and then coverslipped with Permount® (Fisher Scientific, Fair Lawn, NJ) permanent mounting media.

Immunofluorescence was used as an alternate method of visualizing immunoreactivity (IR). Sections were sequentially incubated in primary antibody (Brx; 1:1000) for 48h at 4° C, biotinylated secondary antibody (1:200, biotinylated goat anti-rabbit, Vector) for 1h at room temperature, and Cy-2 or Cy-3-conjugated streptavidin for 1h (1:200; Jackson ImmunoResearch, West Grove, PA). Four 5 minutes washes were performed between each incubation. After a final wash in a saline-free buffer, tissue was mounted onto microscope slides and coverslipped with DAKO® Fluorescent Mounting Medium (DAKO Corp, Carpinteria, CA).

Single Label Slide Mounted ICC

For the pituitary, sections were processed using a modified protocol. Slides were removed from -20° C and allowed to warm to room temperature for 15 minutes on a slide warmer (Fischer Scientific). Slides were rinsed twice in TBS for five minutes. This was followed by 1% hydrogen peroxide in TBS for 10 minutes to inactivate endogenous peroxidase activity. The slides were sequentially incubated in a blocking solution containing 0.1% BSA (Sigma), 5% NGS (Vector) and 0.5% Triton X-100 (EM Scientific) in TBS for 1h, a primary antibody to Brx (1:1000 in 0.05 M TBS plus 0.5% NGS and Triton 0.05% X-100) for 48h at 4° C, biotinylated goat anti-rabbit secondary antibody (1:200; Vector), followed an avidin-biotin-horseradish peroxidase complex (1:400; Vector) for 1.5h. Four 5 minutes washes were performed between each incubation. Immunoreactivity was visualized with 3,3' –diaminobenzidine tetrahydrochloride in TBS. Following extensive washes in TBS, slides were allowed to dry overnight, rinsed in deionized water, dehydrated in alcohols and cleared in xylene, and then coverslipped with Permount® permanent mounting media. As an alternate method, Cy-2 or Cy-3 was used to visualize immunoreactivity using the method described above. All incubations were conducted in a humidity chamber for all slides.

Controls

Positive and negative controls were conducted in the ICC experiments (Figure 10). Negative controls included running the experiment without a primary antibody and running the experiment with a preimmune antisera (serum obtained from the animal prior to exposure to the immunizing Brx-derived peptide; Brx 62

or 67). In addition, pre-absorption studies were also performed. Peptides used to raise the antibodies were allowed to incubate with the antibodies prior to exposure to test samples. Positive controls included running the experiments with antibodies to antigens known to exist in the respective tissue. For example, as a positive control in the pituitary we used an antibody to growth hormone (anti-GH; 1:1000; National Hormone and Peptide Program, NIH, Bethesda, MD). For the brain, the rabbit antibody against the metalloendopeptidase, EC3.4.24.15 (AIS; 1:1000; a kind gift of Dr. A. Ian Smith) was used.

Immunoblotting

Crude Protein Extract Preparation

Crude protein extracts, from fresh frozen tissue were prepared with lysis buffer [0.3 M sucrose, 0.25% soduim deoxycholate, 10mM Tris, 1.5 mM magnesium chloride, 0.5% NP-40, containing protease inhibitors (Complete™, Roche Diagnostics, Mannheim, Germany)] added to the microcentrifuge tube containing the frozen tissue. Tissue was then homogenized using a microcentrifuge sample pestle. Brain, pituitary and peripheral tissue samples were homogenized in a similar fashion but with standard amounts of lysis buffer (100 to 500 µl) for each tissue. After homogenization, the samples were centrifuged at 16,000g for 10 min and the supernatant (crude protein extract) aliquoted and frozen for storage at -20° C. Protein concentration was determined using the Bradford Assay.

Protein Estimation

To standardize the amount of protein added to the electrophoresis gel, all samples in a given Western blot experiment were analyzed at the same time for protein concentration. Serial dilutions of BSA (Promega, Madison, WI) were prepared with lysis buffer to final dilutions of 2 µg/µl, 1.0 µg/µl, 0.5 µg/µl, and 0.25 µg/µl and 0 µg/µl. Fifty microliters of these standards (and dilute samples of crude protein extract) were added to 1.5 ml Bradford Reagent (Sigma Chemical Co., St Louis, MO). Samples were incubated for five minutes at room temperature and mixed prior to measurements on a spectrophotometer (Spectronic Genesys 2). The absorbance was measured at 595 nm. A standard curve was prepared from the BSA standards and the protein concentrations of the crude protein extracts were extrapolated from the standard.

Electrophoresis

Polyacrylamide gels (10%; acrylamide:bis-acrylamide, 37.5:1) in Tris-SDS buffer were purchased from BioRad (Hercules, CA). Twenty µg of crude sample protein extract and an equal volume of loading buffer were mixed in a micro centrifuge tube and heated to 95° C for five minutes then rapidly chilled on ice to unfold proteins and prevent refolding into tertiary structure. Samples were loaded into the gel along with 4 µl of marker mix (BlueRanger® Prestained Protein Molecular Marker Mix, Pierce, Rockford IL). Proteins and markers were run through the gel in a Tris glycine SDS buffer at a constant voltage of 150V until the bromophenol blue marker had migrated to the bottom of the gel (approximately 1h).

Protein Transfer to PVDF Membrane

A PVDF membrane (Invitrogen, Carlsbad, CA) was prepared by immersing in methanol (J.T. Baker, Phillipsburg, NJ) before equilibrating in transfer buffer (25mM tris, 192 mM glycine, 20% methanol). Proteins from the gel were transferred to the PVDF membrane using a transfer apparatus (Bio Rad) set at 80V for 2h.

Immunodetection

The PVDF membrane was incubated in 20 ml blocking solution (ProtoBlock reagent; National Diagnostics, Atlanta, GA) containing sodium azide for 1h. After 2 washes with PBS-Tween (0.1M phosphate buffered saline, pH 7.4, with 0.1% Tween 20; National Diagnostics), the membrane was incubated in 10 ml antibody solution (Brx 62 or Brx 67; 1:10,000) overnight at 4° C with constant shaking. After the primary antibody incubation, the membrane was washed with PBS-Tween and incubated in an HRP-conjugated secondary rabbit antibody (1:500,000; Promega) for 1h at room temperature. After final washes to remove all unbound antibody, the antigen-antibody complex was visualized with a chemilluminence reagent (SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce). The membrane was incubated in the reagent for five minutes at room temperature. Chemiluminescence activity was visualized by exposing the membrane to autoradiograpy film and developing the film on a Kodak M35 X-O MAT photoimager.

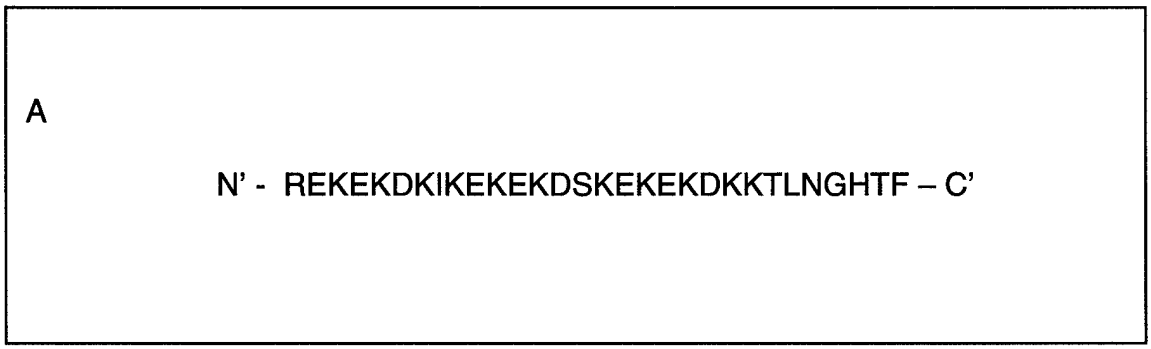
Antibodies

Anti-Brx antibodies were a generous gift of Dr. James Segars (NICHD,NIH). These polyclonal antibodies were prepared by rabbit immunization. Two antibodies were used in this study to characterize the localization and expression of Brx: Brx 62 was raised against a 30-residue peptide derived from the N-terminus of Brx (aa381 to aa410; Figure 9A) and Brx 67 was raised against a 31-residue peptide derived from the C-terminus of Brx (aa942 to aa972; Figure 9B). The peptide sequences used to raise the Brx 62 and Brx 67 antibodies correspond to a portion of the protein sequence located just prior to the DAG region and the C-terminal end of the PH domain, respectively (see Figure 8). A sequence homology search of the NCBI protein database using peptide sequences used to generate Brx 62 and 67 antibodies resulted in homology scores of 88 or better for highly related proteins: Ht-31, Lbc (2001), Brx, and AKAP 13. These results suggest the peptides corresponding to the respective motifs localized within the predicted mouse Brx sequences are not homologus to any other known sequence in the protein database.

Data Analysis

Semi quantitative results were obtained by loading 20 µg of crude protein extract, probing with 10 ml of primary antibody (1:10,000), analyzing all samples together in the same run, and measuring reactivity. To determine relative protein levels, NIH Image software was employed. The films were first scanned and stored as TIFF images. The TIFF files were opened in NIH Image software and the background of the autoradiograph film was measured and subtracted from the image. Next, each individual band was selected and measured. The results

were given in band area (measured in square millimeters) and a mean density. Finally, the area of each band was multiplied by the mean density to obtain relative units. Results are shown as mean values +/- SE, where appropriate. Data from scanned images were compared by analysis of variance. In all cases, $p < 0.05$ was considered to be statistically significant.



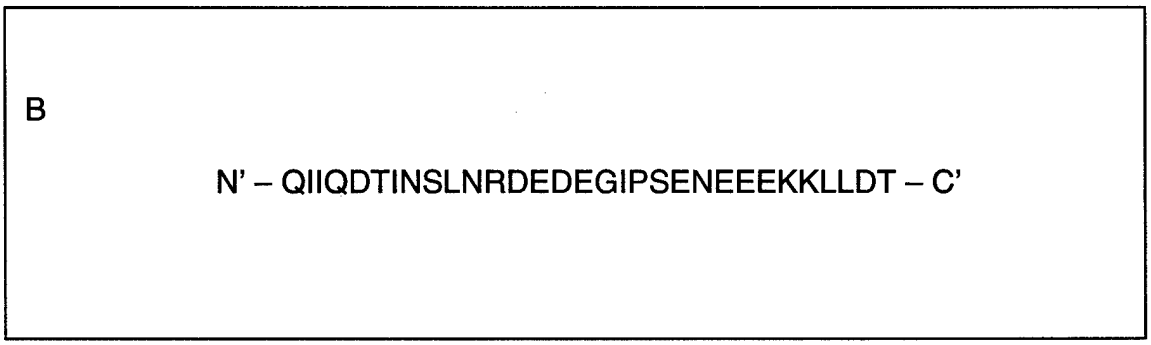


Figure 9: Peptide sequences of Brx 62 (A) and Brx 67 (B) used to generate the antibodies. "A" is derived from the N-terminus of Brx (residues aa381 to aa410) and corresponds to a site just prior to the DAG region. "B" is derived from the C-terminus of Brx (resides aa942- aa972) and corresponds to site located at the C-terminal end of the PH region.

CHAPTER III. RESULTS

Immunocytochemical Localization of Brx in the Adult Brain and Pituitary

Immunoreactive products from representative coronal sections are shown in photomicrographs in Figure 10 and 11. Controls are shown in Figure 10. The results seen in Figure 11a are representative of immunoreactivity seen in the olfactory bulb where immunofluorescence is localized to the vomeronasal nerve. Figure 11b shows staining patterns in the cortex where staining was observed to be most concentrated in layers III and V. Brx expression in the thalamic reticular nucleus is seen in Figure 11c, while Figure 11d shows immunoreactivity in the granular layer of the cerebellum.

The immunoreactivity shown is representative of results obtained with ICC analysis using Brx 67 antibody. The immunoreactivity observed in tissue analyzed with the Brx 62 antibody showed similar staining in the olfactory bulb, but did not result in a staining intensity as clear as did Brx 67 in other areas. The Brx 62 antibody did result in light staining in specific regions of the brain including regions not observed for Brx 67 staining.

Immunocytochemical localization of Brx in the pituitary shows that the immunoreactivity was restricted to the anterior lobe (Figure 11e). No such immunoreactivity was observed in the neural or intermediate lobes of the pituitary. Visual appraisal of randomly selected slides suggests that approximately 20% of cells in the anterior pituitary were observed to express Brx albeit their phenotype remains to be elucidated. Similar distribution of Brx expression was observed in the female as it was in the male. Brx staining at a

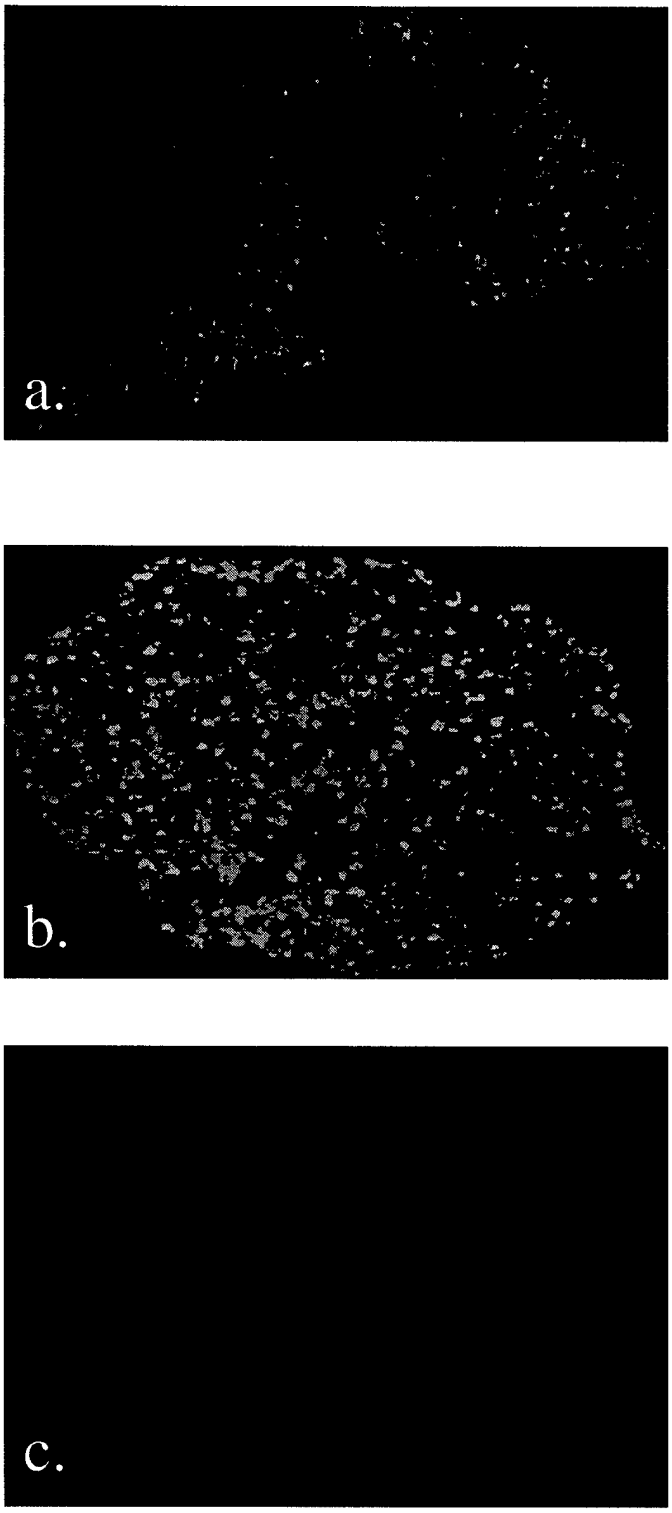


Figure 10: Controls for immunocytochemistry. Photomicrographs were taken with the 5X objective. Pituitary sections showing: a. immunoreactivity to Brx, b. positive control (growth hormone), and c. negative control (no primary antibody). Similar results were observed with pre-immune serum or pre-absorbed antibody.

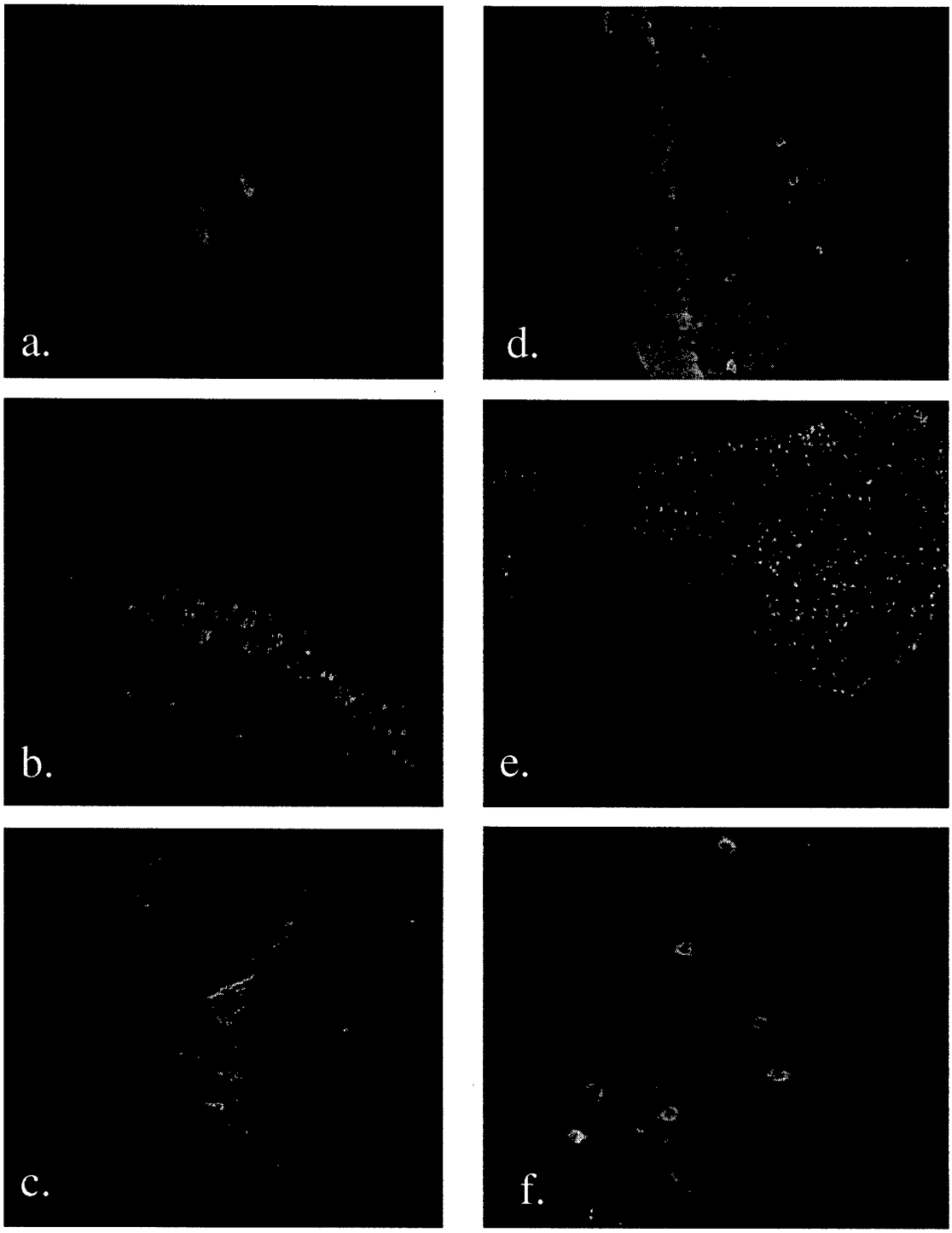


Figure 11: Immunocytochemical localization of Brx in the adult male brain and pituitary. Photomicrographs were taken with the 5X objective (a-e) or 10X objective (f.). Immunoreactivity to Brx 67 antibody is shown in various nervous tissues including: a. olfactory bulb, b. cortex, c. thalamic reticular nucleus, d. cerebellum, e. pituitary. At a higher magnification, the intracellular distribution of Brx suggests membrane association (f.).

high magnification illustrates that the immunofluorescence appears be located at the periphery of the cell (Figure 11f), suggesting a possible intracellular localization associated with the cellular membrane and processes.

Analysis of Brx Expression in the Male by Western Blots

As with the section above on immunocytochemistry, the characterization of Brx with Western analysis tested both Brx 62 and Brx 67 antibodies. The results suggest widespread Brx expression in the brain. The Brx 62 antibody identified an approximately 105 kd protein (Figure 12a). Expression of this protein was observed in the brain (olfactory bulb, cortex, hypothalamus, thalamus, and cerebellum) and peripheral tissues (heart, liver, lung, and testes; data not shown). The Brx 67 antibody identified a primary protein at approximately 70 kd. Like Brx 62, expression of Brx 67 was also seen in the olfactory bulb, cortex, hypothalamus, thalamus, and cerebellum (Figure 12b).

Sex Differences in Brx Expression in the Cortex and Pituitary

To determine the relative expression of Brx in the cortex of male and female mice, semi-quantitative Western analysis was conducted. The results showed a significant difference in expression of Brx in the cortex of males and females (2066 +/- 71 and 2530 +/- 138 relative units, respectively; $p<0.05$; $n = 4$ per group; Figure 13). To determine if gender differences in Brx expression in the pituitary gland exist, pituitaries from the same male and female mice were compared (Figure 14). The results indicate that Brx is expressed in greater

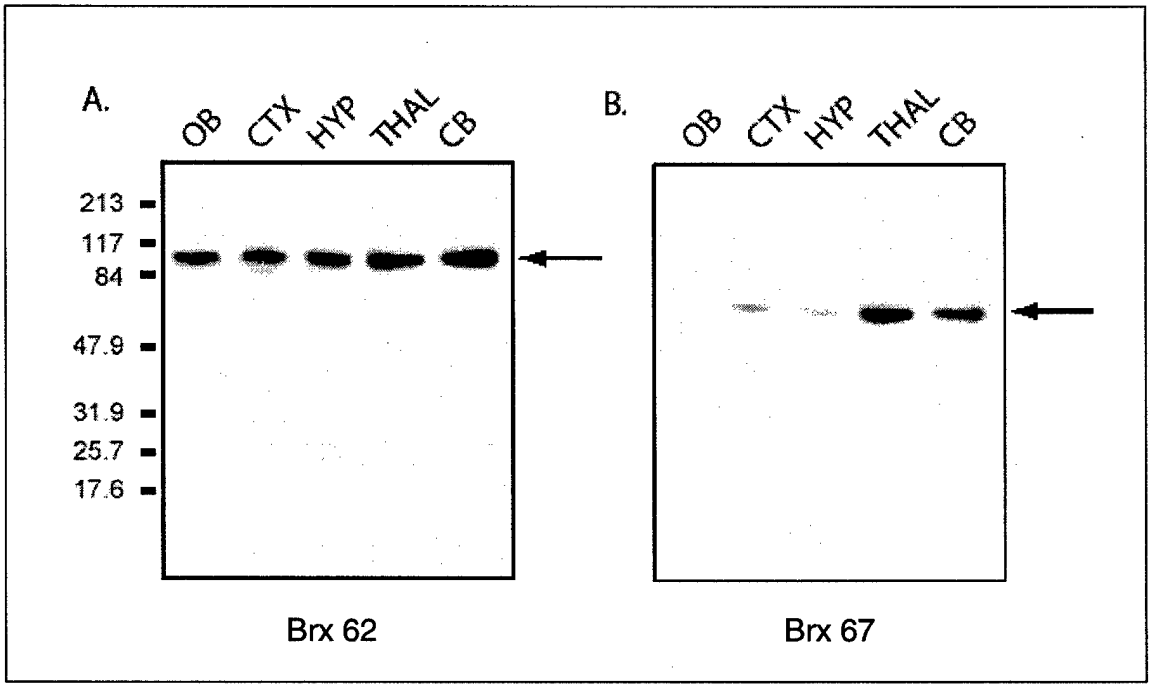


Figure 12: Western analysis of Brx expression in male mouse brain. Brx is expressed in olfactory bulb, cortex, hypothalamus, thalamus and cerebellum. Gels were loaded with 20 µg of protein extract and analyzed with A. Brx 62 and B. Brx 67 antibodies.

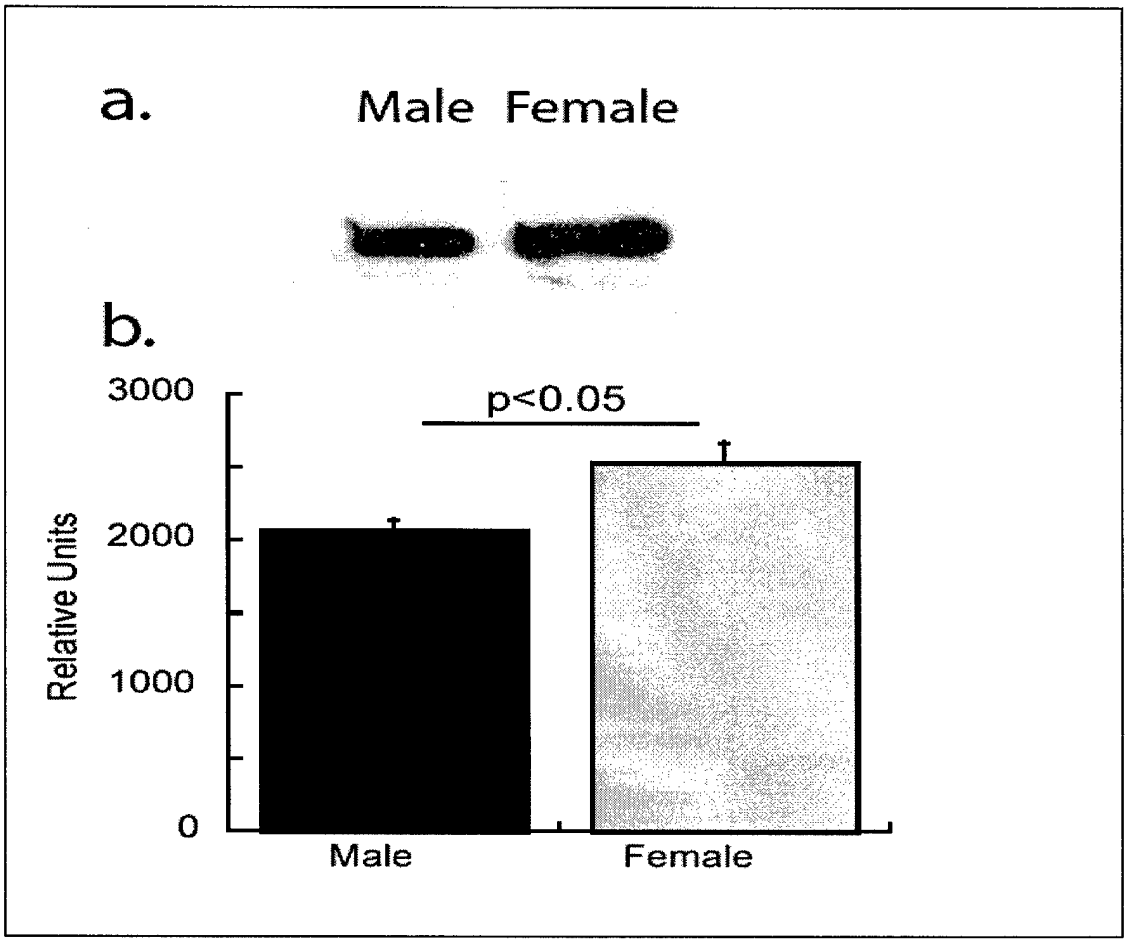


Figure 13: Brx expression in male and female cortex. Cortex from female mice show higher levels of expression than males. A. is a representative Western blot showing Brx expression in the cortex of a male and a female mouse. B. Relative expression of Brx in cortex of male and female mice (mean+/- se). The western blots were analyzed using the NIH Image software. Males average 2066 +/- 71 relative units, while females averaged 2530 +/- 138 relative units ($p<0.05$; $n = 4$ per group).

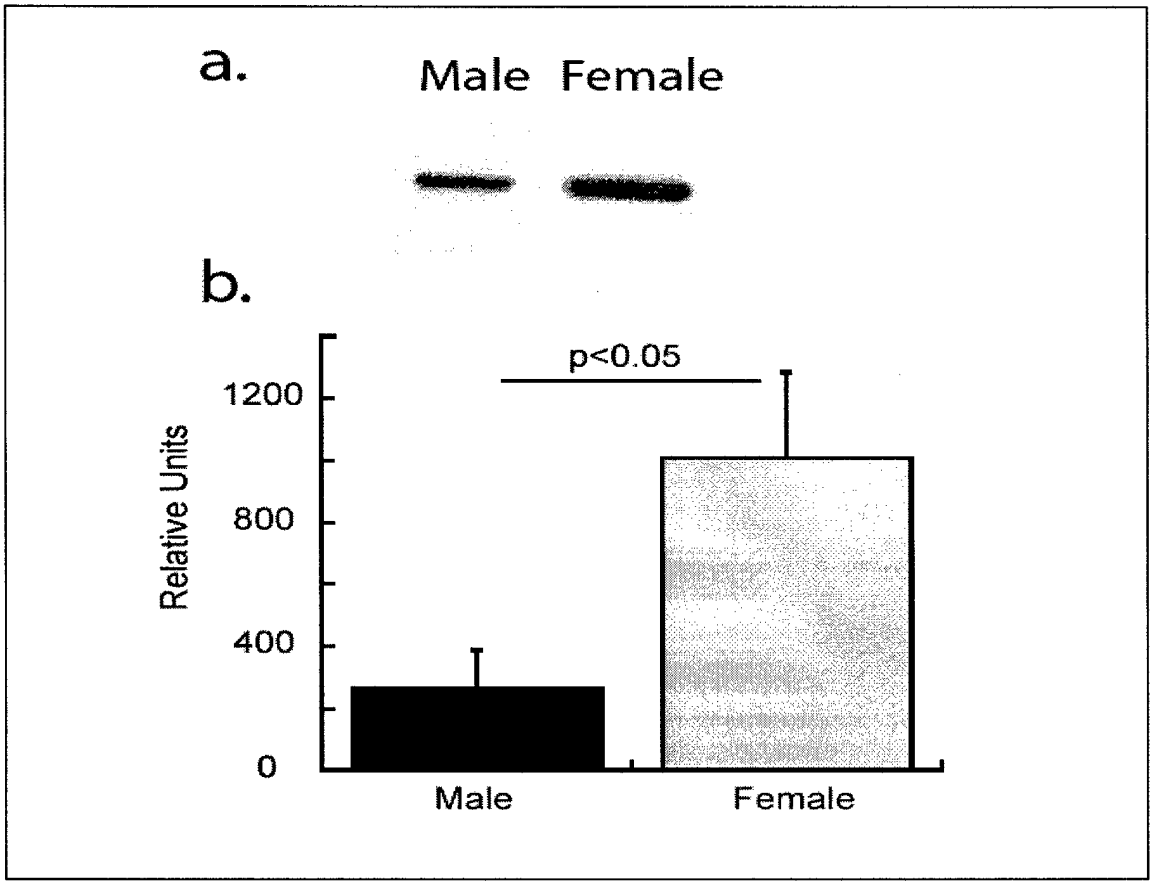


Figure 14: Brx expression in male and female pituitary gland. Pituitary from female mice show higher levels of expression than males. A. is a representative Western blot showing Brx expression in the pituitary of a male and a female mouse. B. Relative expression of Brx in cortex of male and female mice (mean+/- se). The western blots were analyzed using the NIH Image software. Males average relative expression was 265 +/- 124 relative units, while females averaged 1012 +/- 275 relative units ($p<0.05$; n = 4 per group).

levels in female mice (1012 +/- 275 relative units) than in male mice (265 +/-124 relative units; $p < 0.05$; $n = 4$ per group).

Estrogen Regulation of Brx Expression

To determine the possible effect of estrogen on Brx expression in the pituitary, 7-day ovariectomized mice were randomly assigned to receive an administration of estrogen (20 µg estradiol benzoate; s.c.) or vehicle (control). The results show that estradiol benzoate administration did not affect Brx expression (572 +/- 192 relative units and 708 +/- 122 relative units, respectively; $p > 0.10$; $n = 4$ per group; Figure 15).

Extracts from the cortex of both EB-treated and ovariectomized control mice were also analyzed by Western blot. The expression of Brx in the cortex was not different between EB-treated and vehicle-treated mice (2655 +/- 228 relative units vs. 3115 +/- 153 relative units, respectively; $p >= 0.10$; Figure 16).

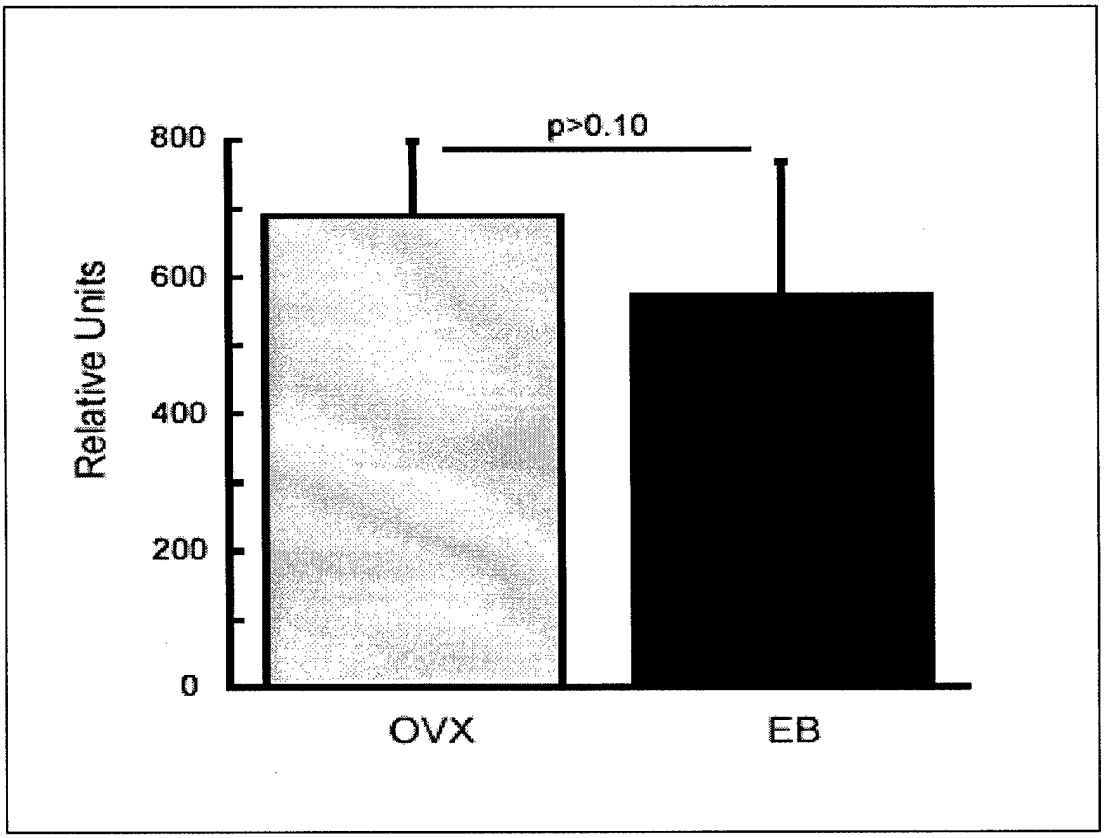


Figure 15. Estrogen regulation of Brx expression in pituitary gland. Brx expression was compared between ovariectomized mice that received a 20µg injection of estradiol benzoate (s.c.) and those who had received an oil vehicle. No significant differences were observed (708 +/- 122 relative units and 572 +/- 192 relative units, respectively; $p > 0.10$; $n = 4$ per group).

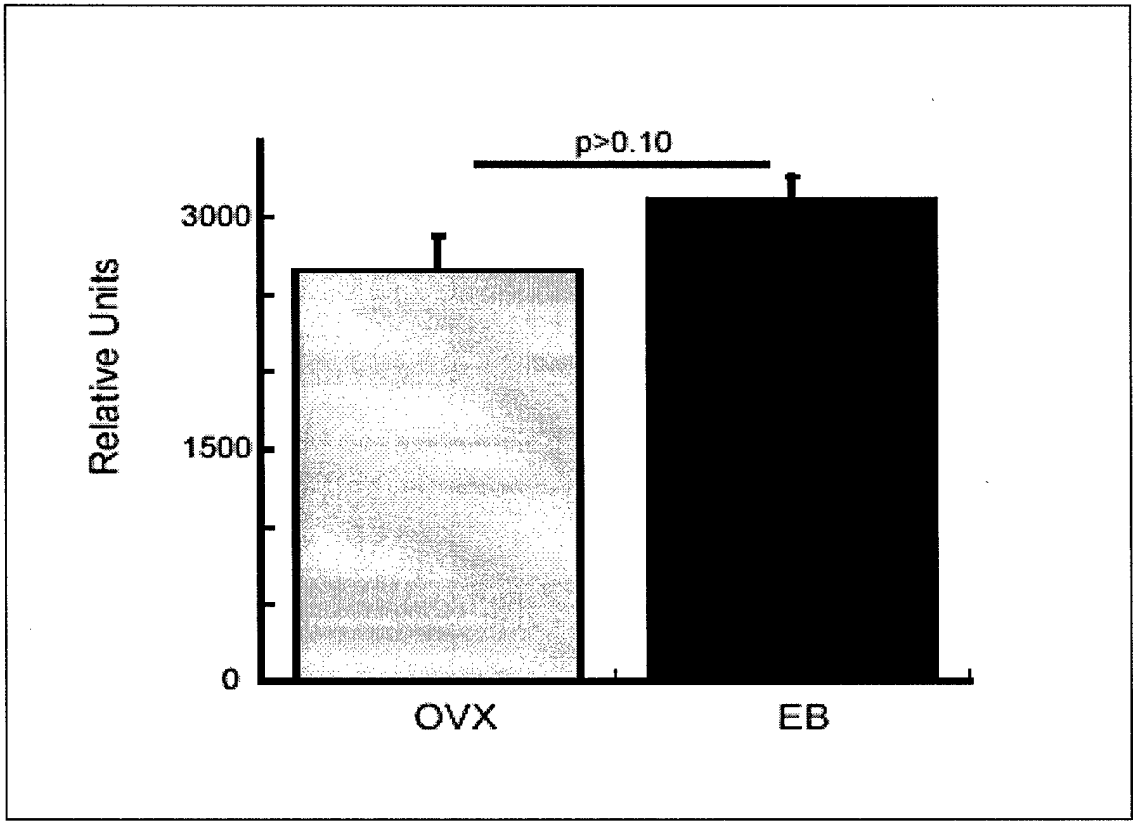


Figure 16. Estrogen regulation of Brx expression in the cortex. Brx expression was compared between ovariectomized mice that received a 20μg injection of estradiol benzoate (s.c.) and those who had received an oil vehicle. No significant difference were observed (2655 +/- 228 relative units and 3115 +/- 153 relative units, respectively; $p > 0.10$; $n = 4$ per group).

CHAPTER IV. DISCUSSION

The effects of estrogen in the brain and pituitary are significant and have been shown to regulate reproductive and other non-reproductive functions and behaviors [20]. Estrogen may also provide neuroprotection from injury and neurodegeneration. Consequently, understanding the function of estrogen and its receptors is important. The role of the ER-associated protein, Brx, in mediating estrogenic effects is poorly understood. The goal of this thesis was to characterize the expression and localization of Brx in brain and pituitary gland of adult mice.

The results of this study showed Brx expression in the adult male mouse brain, and provides the first evidence of Brx expression in the pituitary of both male and female mice. In the male brain, Brx is localized in the vomeronasal organ (VNO), cortex (layers III and V), the thalamic reticular nucleus (TRN), and the granular layer of the cerebellum (Figure 11). In rodents, seven transmembrane G-coupled receptors in the VNO are believed to be activated by pheremones to affect behavior [87, 88]. The GEF region of Brx and its presence in this locale may suggest an important role for Brx in mediating signaling pathways important for sexual behavior. It is interesting to note that within the cortex, Brx was found in layers III and V. Neurons in layer III project primarily within the cortex with some projecting contralaterally. Cortical layer V is a major projection route out of the cortex. The thalamic reticular nucleus is important in attention, performance, vigilance and memory. The TRN plays a crucial role in modifying patterns of activity that can reach the cerebral cortex from the

thalamus [91] and serves as a nexus where cortical and thalamic nuclei can interact [92]. In addition, the TRN may be important in prolactin secretion since showed the strongest signal of prolactin-releasing peptide in the brain [94]. These regions of the brain that contain Brx-positive neurons were previously shown to colocalize with glutamate decarboxylase (GAD) [81]. These regions have a high density of GABAergic neurons which exert local inhibitory effects [89,90].

Interestingly the regions of the brain that express Brx also appear to coincide with a subset of the regions that express ER-β [80]. Taken together, the localization of Brx suggests its involvement in ER-β-mediated signaling in a subset of ER-β expressing cells in the central nervous system. A study of Brx expression in ER-α and ER-β knock-out mice would likely shed light to this question. That Brx appeared to localize in regions of the brain that express ER-β supports a previous study showing that Brx activation of ER-β is mediated by p38 MAPK [68].

In the pituitary, Brx is localized in the anterior pituitary but not the intermediate or neural lobes. It is well established that the pituitary is a major target for estrogen [82]. While more studies are needed to determine the phenotype of these Brx-positive cells, we speculate that Brx is localized in lactotrophs. There are two reasons to suggest this as a possibility. First, pituitary lactotrophs are a major target of estrogen among the anterior pituitary cells [47, 83] with 50% of lactotrophs shown to contain ER-α and 30% to shown to contain ER-β. Secondly, the distribution of Brx-positive cells in a scattered

pattern in the lateral anterior pituitary, but not the medial portion, coincides with the distribution of lactotrophs [47]. Future studies include the identification of the phenotype of these Brx-positive cells using double-labeling immunocytochemistry and/or a combination of *in situ* hybridization histochemistry with immunocytochemistry.

An interesting observation of the cellular distribution of Brx suggests that the localization of this protein is associated with the plasma membrane (Figure 11F). This observation is supported by two putative structural motifs located in the Brx structure: the PH domain and the diacylglycerol (DAG) binding site. DAG is involved in membrane lipid signaling [73]. It is one of the products released when phospholipase C cleaves phosphatidylinositol 4,5-bisphophate (PIP_2) in signaling pathways. DAG is hydrophobic and remains at the cell membrane, while the other product, inositol 1,4,5-triphosphate (IP3) is released to bind to its receptor in the endoplasmic reticulum causing the opening of Ca^{++} ion channels, leading to activation of protein kinase C (PKC), among other things. The accumulation of DAG causes PKC to migrate from the cytosol to the cell membrane where it becomes activated. The PH domain (seen in Dbl-family proteins) is also affiliated with plasma membrane signaling [78, 79]. Collectively, the anatomical distribution of Brx suggests that it may be involved in signaling complexes mediating ER function. It is interesting to note that pituitary-specific truncated estrogen receptor products (TERPs) exist in 4-6 fold greater concentrations compared to the full length ER's [47] and that the truncation of ERs through a splice mechanism have been suggested to shuttle the ER to the

plasma membrane [95,96]. It is interesting to speculate that Brx may be associated with signaling pathways mediated by TERPs.

The immunoreactivity analyzed by immunocytochemistry using the Brx 62 antibody was different from that seen with the Brx 67 antibody. Brx 62 demonstrated light staining and some cells stained were in specific regions of the brain where Brx 67 antibody staining was not observed. It is possible that the sequence containing the Brx 62 peptide may represent an epitope not accessible to the antibody when Brx is in its tertiary structure or in association with the plasma membrane. Antigen retrieval or alternative fixation methods may be needed to improve visualization. Another possible reason for this observation may be the phosphorylation state of Brx. The Brx 62 sequence contains threonine and serine (see Figure 9), which are known to be phosphorylation sites. Addition of the phosphate group may interfere with antibody binding. However, this is unlikely because the antibodies bind the antigen site in the immunoblots.

Interestingly, Western blot analysis showed that both Brx 62 and Brx 67 antibodies identified different molecular weight proteins. This is consistent with previous studies showing splice variants of Brx in tissues including the proto-Lbc (~102 kd) [65]. In this study, Brx 62 identified a protein that is approximately ~105 kd. Another study suggested both Brx and Lbc were splice variants of an even larger AKAP protein designated Ht-31 [71]. Using cDNAs from Brx and Lbc, and a partial Ht31 cDNA the composite Ht31 cDNA length was determined to be 8442 bp (corresponding to a ~309 kd protein). Ht31, proto-Lbc, Brx and

AKAP 13 are all mapped to the same gene on chromosome 15 q24-25 in the human genome. The heteronuclear cDNA contains 38 exons, 98-100 % homologous to 38 blocks of genomic DNA. Also, antibodies to portions of Ht31 not part of the Brx or proto-lbc transcripts demonstrated multiple banding patterns, further suggesting splice variation in this gene. Numerous additional splice variants of Ht-31 have been shown in various malignant tissues. It is unclear whether the splice variants of Ht31 are cell-line specific or malignancy-specific. However, splice variation of this gene within the same cell would account for the different-sized proteins detected by Brx 62 and Brx 67. Alternatively, Brx may be spliced into smaller peptides in the process of signaling, as is the case with the Notch protein [84]. It is also possible that the peptides detected were degradation products of Brx, although significant efforts to avoid degradation were employed. These include the use of protease inhibitors and minimizing the time tissue was at temperatures favorable for degradation. The proteins detected by Brx 62 and Brx 67 were much smaller (~105 kd and ~70 kd respectively) than the full-length human Brx protein (~170 kd). In this study, only the soluble fraction was used in the immunoblotting. It is conceivable that the full length Brx is membrane-associated, consistent with its structural motifs, and remained in the particulate fraction. Since the rodent equivalent of Brx has yet to be characterized, it is possible that the mouse Brx gene is unlike the human form. At least one study has shown that AKAP proteins can be nearly twice as large in humans than in rodents [85]. Until the complete

mouse Brx is sequenced, and resulting protein products established, this remains to be elucidated.

The present results show that female mice have higher Brx expression than males in the pituitary and cortex (Figures 13,14). The differences were most notable in the pituitary, where there is a four-fold greater expression in females than males. Interestingly, this corresponds to the marked sex differences in lactotrophs in the anterior pituitary of male and female mice. Females were shown to contain more prolactin-producing cells than males by a factor of approximately four. This observation is additional support for the idea that Brx-expressing cells may be co-located with lactotrophs. The finding that Brx expression is higher in the cortex of females than males correlates with greater levels of estrogen receptor in the brain of females than males [86].

Sexual dimorphic differences in Brx expression and its structural motif suggest that Brx might be regulated by E2. However, no differences in the expression of Brx protein in the cortex and pituitary of estrogen-treated mice were observed here. These findings indicate that the regulation of Brx may be developmentally controlled and its expression is independent of circulating estrogen levels. On the other hand, it is possible that though no differences in Brx expression were observed in the pituitary and cortex of mice after ovariectomy and estrogen treatment, there may be other area of the brain where expression is modified.

Summary

The studies in this thesis described Brx localization in the male brain and the anterior pituitary. These studies also showed that females express Brx at a higher level in the pituitary and cortex than do males. In the female, Brx expression is not regulated by estrogen since ovariectomized animals treated with estradiol benzoate did not increase expression of Brx. These results suggest that Brx is developmentally regulated. Its role in modulating intracellular signaling pathways remain to be elucidated. Future studies evaluating the phenotype of Brx-positive cells will enhance our understanding of its function. In addition, the structure of this novel protein in the mouse needs to be resolved.

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